# Zinc ameliorates human aortic valve calcification through GPR39 mediated ERK1/2 signaling pathway

Cardiovascular Research

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--Manuscript Draft--

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| **Abstract:** | Aims: Calcific aortic valve disease is the most common heart valve disease in the Western world. It has been reported that zinc is accumulated in calcified human aortic valves. However, whether zinc directly regulates calcific aortic valve disease is yet to be elucidated. The present study sought to determine the potential role of zinc in the pathogenesis of calcific aortic valve disease.<br>Methods and Results: Using a combination of a human valve interstitial cell calcification model, human aortic valve tissues and blood samples, we report that 20 μM zinc supplementation attenuates human valve interstitial cell (hVIC) in vitro calcification, and that this is mediated through inhibition of apoptosis and osteogenic differentiation via the zinc sensing receptor GPR39-dependent ERK1/2 signaling pathway. Furthermore, we report that GPR39 protein expression is dramatically reduced in calcified human aortic valves, and there is a significant reduction in zinc serum levels in patients with calcific aortic valve disease. Moreover, we reveal that 20 |

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| | μM zinc treatment prevents the reduction of GPR39 observed in calcified human valve interstitial cells. We also show that the zinc transporter ZIP13 and ZIP14 are significantly increased in hVICs in response to zinc treatment. Knockdown of ZIP13 or ZIP14 significantly inhibited hVIC in vitro calcification and osteogenic differentiation. Conclusions: Together, these findings suggest that zinc is a novel inhibitor of calcific aortic valve disease, and report that zinc transporter ZIP13 and ZIP14 are important regulators of hVIC in vitro calcification and osteogenic differentiation. Zinc supplementation may offer a potential therapeutic strategy for calcific aortic valve disease.<br>Translational Perspective<br>This study reports that the zinc sensing receptor GPR39 expression is decreased in calcified human aortic valve tissues and there is a significant reduction in zinc serum levels in patients with calcific aortic valve disease. Zinc treatment attenuates hVIC in vitro calcification through inhibition of apoptosis and osteogenic differentiation via GPR39-dependent ERK1/2 signaling pathway. Zinc transporter ZIP13 and ZIP14 are important regulators of hVIC in vitro calcification and osteogenic differentiation. Zinc supplementation is a potential therapeutic strategy for calcific aortic valve disease. |

## Abstract

**Aims:** Calcific aortic valve disease is the most common heart valve disease in the Western world. It has been reported that zinc is accumulated in calcified human aortic valves. However, whether zinc directly regulates calcific aortic valve disease is yet to be elucidated. The present study sought to determine the potential role of zinc in the pathogenesis of calcific aortic valve disease.

**Methods and Results:** Using a combination of a human valve interstitial cell calcification model, human aortic valve tissues and blood samples, we report that 20 μM zinc supplementation attenuates human valve interstitial cell (hVIC) *in vitro* calcification, and that this is mediated through inhibition of apoptosis and osteogenic differentiation via the zinc sensing receptor GPR39-dependent ERK1/2 signaling pathway. Furthermore, we report that GPR39 protein expression is dramatically reduced in calcified human aortic valves, and there is a significant reduction in zinc serum levels in patients with calcific aortic valve disease. Moreover, we reveal that 20 μM zinc treatment prevents the reduction of GPR39 observed in calcified human valve interstitial cells. We also show that the zinc transporter ZIP13 and ZIP14 are significantly increased in hVICs in response to zinc treatment. Knockdown of ZIP13 or ZIP14 significantly inhibited hVIC *in vitro* calcification and osteogenic differentiation.

**Conclusions:** Together, these findings suggest that zinc is a novel inhibitor of calcific aortic valve disease, and report that zinc transporter ZIP13 and ZIP14 are important regulators of hVIC *in vitro* calcification and osteogenic differentiation. Zinc supplementation may offer a potential therapeutic strategy for calcific aortic valve disease.

**Translational Perspective**

This study reports that the zinc sensing receptor GPR39 expression is decreased in calcified human aortic valve tissues and there is a significant reduction in zinc serum levels in patients with calcific aortic valve disease. Zinc treatment attenuates hVIC *in vitro* calcification through inhibition of apoptosis and osteogenic differentiation via GPR39-dependent ERK1/2 signaling pathway. Zinc transporter ZIP13 and ZIP14 are important regulators of hVIC *in vitro* calcification and osteogenic differentiation. Zinc supplementation is a potential therapeutic strategy for calcific aortic valve disease.


**Key words**: Zinc, valve interstitial cell calcification, apoptosis, ERK1/2, GPR39

Guangzhou, 08.03.2020

**Cardiovascular Research- CVR-2019-1285R2**

Dear, Prof. Magnus Bäck

We are pleased to submit our revised manuscript 'Zinc ameliorates human aortic valve calcification through GPR39 mediated ERK1/2 signaling pathway' for your kind consideration. We confirm that this manuscript is not submitted elsewhere or under consideration for publication. All authors have read and agreed with the submission of the manuscript.

We thank the reviewers for their highly constructive comments and suggestions, and we have revised our manuscript accordingly. This has entailed editing and adding new text and data. New text is highlighted in red font. Below we have listed each of the reviewers' comments and provided our detailed response. We believe that our manuscript is much improved, and we hope that it is now considered suitable for publication in Cardiovascular Research.

Yours sincerely

On behalf of all authors

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**Cardiovascular Research- CVR-2019-1285R2**

**Authors' response to reviewers' comments**

**Reviewer #1:**

**Reviewer comment 1:**

Just one minor thing I can't help but mention-in the revised discussion:

'Our data suggest that knockdown of ZIP13 or ZIP14 may reduce hVIC calcification through attenuation of osteogenic transition. In addition, ALP, a key regulator of cardiovascular calcification, have been reported to contain two zinc-binding sites, which are essential for its catalytic activity37. We have shown that knockdown of ZIP13 or ZIP14 decreased cytosolic zinc concentrations, which may lead to reduced catalytic activity of ALPL, thereby inhibiting calcification.'

I find this statement highly speculative and not very straightforward as a mechanism. ALP could be expected to be mediating pro-calcific effects in the extracellular space, if the reduction of intracellular zinc would impair enzyme function is rather far stretched. My recommendation would be to omit that and to state "The mechanisms underlying the anti-calcific effects of ZIP13 or ZIP14 silencing are currently elusive and require further study." Or similar, if the authors choose to do so.

**Authors' response:** We thank the reviewer's comments and suggestions. We have revised our text accordingly.

Discussion: Line 504-506

'The mechanisms underlying the anti-calcific effects of ZIP13 or ZIP14 silencing are currently elusive and require further study.'

**Reviewer #3:**

**Reviewer comment 1:** Please provide echo data for patients in Suppl. Table II.

**Authors' response:** We are extremely sorry that we did not record the echo data for patients in Suppl. Table II at the time of sample collection. In the present study, alizarin red staining was used to measure calcium deposition in these aortic valve samples. As shown in Figure 6A, positive staining of alizarin red was observed in calcified aortic valves, while no staining of alizarin red was seen in non-calcified aortic valves.

**Reviewer comment 2:** In Suppl. Table III, according to the legend ARA would be «aortic

regurgitation area» in cm2; the meaning is not clear. If AR was significant, it should be documented by the regurgitant fraction and ERO. Please clarify as if these patients had significant AR including these data makes probably no sense. It appears that this group of patients had aortic valve sclerosis, it should be underlined in the result section.

**Authors' response:** We thank the reviewers' comments. In china, aortic regurgitation area is the routinely used index in clinical practice to evaluate the severity of AR, and the regurgitant fraction and ERO are not routinely measured by echo in the hospitals in china. We are sorry that we cannot collect these two specific variables. We agree with the reviewer's comments, and we have removed ARA «aortic regurgitation area» from Suppl. Table III in our revised manuscript. We agree with the reviewer's comments that this group of patients had aortic valve sclerosis, and we have highlighted it in the results section as below.

Results: Line 413-416

'These patients had aortic valve sclerosis, which is an early stage of CAVD and characterized by thickening and calcification of the aortic valve without obstruction of ventricular outflow (Supplementary Figure I and Supplementary material Table III).'

**Reviewer comment 3:** Fluorescent assay and IF in figure 5H (for Zn) and 7A need to be quantified.

**Authors' response:** We have quantified Fluorescent assay and IF in figure 5H (for Zn) and 7A in our revised manuscript. These new data are presented as Figure 5I and revised Figure 7A (lower panel).

# Zinc ameliorates human aortic valve calcification through GPR39 mediated ERK1/2 signaling pathway

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**Short title**: Novel role of zinc in aortic valve calcification

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**The category of the manuscript:** Original Article

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**List of Abbreviations**

CAVD- Calcific aortic valve disease, LVEF- Left ventricular ejection fraction, RUNX2- Runt-related transcription factor 2, VICs- Valve interstitial cells, TGFβ1- Transforming growth factor -β1, TRAIL- TNF-related apoptosis-inducing ligand, NF-κB- Nuclear factor κB, VSMCs- Vascular smooth muscle cells, GPR39- G protein coupled receptor 39, TNFAIP3- TNF-α–induced protein 3, TPEN- N,N,N',N'-tetrakis (2-pyridinylmethyl)-1,2-ethanediamine, BCA- Bicinchoninic acid, PMSF- Phenylmethylsulfonyl fluoride, ALPL- Alkaline phosphatase, MSX2- Msh Homeobox 2, BMP- Bone morphogenetic protein, CKD- Chronic kidney disease.

## Abstract

**Aims:** Calcific aortic valve disease is the most common heart valve disease in the Western world. It has been reported that zinc is accumulated in calcified human aortic valves. However, whether zinc directly regulates calcific aortic valve disease is yet to be elucidated. The present study sought to determine the potential role of zinc in the pathogenesis of calcific aortic valve disease.

**Methods and Results:** Using a combination of a human valve interstitial cell calcification model, human aortic valve tissues and blood samples, we report that 20 μM zinc supplementation attenuates human valve interstitial cell (hVIC) *in vitro* calcification, and that this is mediated through inhibition of apoptosis and osteogenic differentiation via the zinc sensing receptor GPR39-dependent ERK1/2 signaling pathway. Furthermore, we report that GPR39 protein expression is dramatically reduced in calcified human aortic valves, and there is a significant reduction in zinc serum levels in patients with calcific aortic valve disease. Moreover, we reveal that 20 μM zinc treatment prevents the reduction of GPR39 observed in calcified human valve interstitial cells. We also show that the zinc transporter ZIP13 and ZIP14 are significantly increased in hVICs in response to zinc treatment. Knockdown of ZIP13 or ZIP14 significantly inhibited hVIC *in vitro* calcification and osteogenic differentiation.

**Conclusions:** Together, these findings suggest that zinc is a novel inhibitor of calcific aortic valve disease, and report that zinc transporter ZIP13 and ZIP14 are important regulators of hVIC *in vitro* calcification and osteogenic differentiation. Zinc supplementation may offer a potential therapeutic strategy for calcific aortic valve disease.

**Translational Perspective**

This study reports that the zinc sensing receptor GPR39 expression is decreased in calcified human aortic valve tissues and there is a significant reduction in zinc serum levels in patients with calcific aortic valve disease. Zinc treatment attenuates hVIC *in vitro* calcification through inhibition of apoptosis and osteogenic differentiation via GPR39-dependent ERK1/2 signaling pathway. Zinc transporter ZIP13 and ZIP14 are important regulators of hVIC *in vitro* calcification and osteogenic differentiation. Zinc supplementation is a potential therapeutic strategy for calcific aortic valve disease.


**Key words**: Zinc, valve interstitial cell calcification, apoptosis, ERK1/2, GPR39

## 1. Introduction

Calcific aortic valve disease (CAVD), the most common heart valve disease, is a major public health problem in the Western world. It is a chronic disorder that is characterized by progressive fibrocalcific valve thickening and ventricular function impairment, subsequently leading to left ventricular outflow obstruction[1]. Traditional cardiovascular drugs such as statins are unable to prevent the progression of CAVD. Currently, the viable treatments for patients with severe CAVD are surgical valve replacement or transcatheter aortic valve implantation with the prosthetic valves[2]. However, these treatments are invasive, costly and risky for elderly adults, and it may lead to more severe complications including blood clots, infections, and heart attack. In addition, the prosthetic valves have limited durability, and undergo structural degeneration and calcification[3]. A better understanding of the pathophysiology of CAVD is therefore critical for the development of novel therapeutic strategies to slow or reverse the progression of CAVD.

Accumulating evidence indicates that CAVD is an actively regulated and progressive disease, which shares many similarities to physiological bone mineralization. Osteogenic genes like runt-related transcription factor 2 (RUNX2) have been shown to be up-regulated in calcified human aortic valves[4]. Patients with chronic kidney disease (CKD) have increased circulating calcium (Ca) and phosphate (Pi) levels, and are highly susceptible to CAVD[5, 6]. Accordingly, we and others have previously reported that valve interstitial cells (VICs), the most abundant cell type in the aortic valve, undergo osteogenic differentiation in response to high Ca and Pi mimicking that observed in CKD patients[7, 8]. Based on these observations, it is now recognized that the osteogenic differentiation of VICs plays an important role in the development of CAVD. Furthermore, cytokines involved in calcification including the transforming growth factor-β1 (TGFβ1) and TNF-related apoptosis-inducing ligand (TRAIL) are overexpressed in calcific aortic valves[9, 10]. These cytokines promote aortic valve calcification through induction of VIC apoptosis[9, 10]. Conversely, pro-survival signals like ATP have been shown to prevent aortic valve calcification[11]. These studies suggest that apoptosis is also involved in the pathogenesis of CAVD.

Zinc is an important micronutrient for health, which modulates numerous cellular processes including DNA and protein synthesis, enzyme activity, and intracellular signaling. Zinc deficiency is associated with cardiovascular disease. It has been shown that zinc deficiency enhances vascular inflammation and atherosclerotic plaque formation in ApoE knockout mice[12]. Accordingly, zinc supplementation attenuates a high cholesterol diet-induced atherosclerosis in rabbits[13]. Zinc has also been reported to inhibit abdominal aortic aneurysm formation in mice through induction of zinc finger protein A20-mediated suppression of nuclear factor κB (NF-κB) pathway[14]. Furthermore, a recent study has demonstrated that zinc ameliorates phosphate-induced osteogenic transition of vascular smooth muscle cells (VSMCs) and vascular calcification through the G protein coupled receptor 39 (GPR39)-dependent induction of TNF-α–induced protein 3 (TNFAIP3) and subsequent suppression of the NF-kB pathway[15]. Interestingly, zinc has been shown to be accumulated in calcified human aortic valves[16]. However, the role of zinc in the development

of CAVD has not been previously investigated.

In the present study, we have performed detailed analysis of clinical samples from patients with CAVD in conjunction with *in vitro* calcification studies in hVICs to address the possible effects of zinc and zinc transporters on aortic valve calcification and the underlying mechanisms through which zinc and zinc transporters may regulate CAVD.

## 2. Experimental procedures

### 2.1 Human samples

This study complies with the Declaration of Helsinki and approved by The Research Ethics Committee of Guangdong Provincial People's Hospital and Guangzhou Medical University (Ref No: GDREC2019433H). 34 tricuspid aortic valves from patients with CAVD (25 males/9 females) and 4 non-calcified aortic valves from patients with aortic valve prolapse (4 males) undergoing valve replacement surgery were collected at Guangdong Provincial People's Hospital (Guangzhou, China). Marked increase in echogenicity of aortic valves measured by echocardiograms was considered as CAVD by clinical doctors. A representative still image of echocardiograms of a patient with CAVD is shown in Supplementary Figure I. Patients with a history of rheumatic disease, congenital valve disease, and infective endocarditis were excluded. Human blood samples were also collected from 15 healthy volunteers and 15 patients with CAVD at Guangdong Provincial People's Hospital (Guangzhou, China). Informed consent was obtained from all patients. Serum was obtained by immediate centrifugation and stored at -80°C. Clinical characteristics of the patients used in the present study are summarized in Supplementary material Table I-III. The methodology, conduct, and reporting of this study were in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement initiatives for case-control studies[17]. STROBE recommendations for reporting case-control studies are available as Supplementary material Table IV.

### 2.2 Measurement of zinc serum levels

Zinc concentrations were analyzed by an automatic biochemical analyzer (Chemray 240) with the Zinc Assay Kit (Changchun Huili Biotech, C017) according to the manufacturer's instructions.

### 2.3 Isolation of Human Valve Interstitial Cells (hVICs)

hVICs were isolated from non-calcified areas of the valves from patients with CAVD, and the purity of cell preparation was characterized as previously described[8]. Briefly, non-calcified areas of valve leaflets were dissected, incubated with 1 mg/ml trypsin (Gibco, 12605-010) for 10 min and washed in HBSS buffer (Hyclone, SC30588.01) to remove valve endothelial cells. The valve tissues were then digested in 250 U/ml type II collagenase solution (Worthington, 47D17411A) at 37°C for 7 hrs. The cells subsequently obtained were re-suspended in growth media consisting of α-MEM supplemented with 10% FBS (Gibco, 16000-044), 100 U/mL of penicillin (HyClone, SH40003.01), and 100 mg/ml of streptomycin (Hyclone, SV30010), and plated onto a 25 $cm^2$ flask coated with 0.25 $\mu g/cm^2$ type I collagen

(Gibco, A1048301). Cells used for experiments in the present study were between 2-4 passages.

### 2.4 Induction of hVIC *in vitro* calcification

hVICs were seeded at the density of $1.0x10^5$ cells/well in 6-well plates and cultured with growth media. Calcification was induced as previously described[8]. Briefly, hVICs were grown to confluence and treated with control (1.0 mM Pi/1.8mM Ca) or calcifying media (50 μg/mL ascorbic acid/2.5 mM Pi/2.7 mM Ca) for up to 7 days. Pi was prepared as a combination of $NaH_2PO_4/Na_2HPO_4$, pH=7.4. To evaluate the effects of zinc on hVIC *in vitro* calcification, the indicated concentrations (0-20 μM) of $ZnSO_4$ (Sigma, Z2051), the specific zinc chelator N,N,N',N'-tetrakis (2-pyridinylmethyl)-1,2-ethanediamine (TPEN) (Sigma, P4431), $ZnCl_2$ (Sigma, 746355), albumin (Sigma, SRP6516) and ERK1/2 inhibitor PD98059 (Cell Signaling Technology, 9900) were added to the culture media. The media was changed every third/fourth day.

### 2.5 Determination of hVIC *in vitro* calcification

Calcium deposition was determined by alizarin red staining and calcium colorimetric assay. Briefly, the cells were washed twice with cold PBS, fixed with 4% paraformaldehyde (PFA) for 10 min, stained with 2% alizarin red (pH 4.2) for 10 min at room temperature, and photographed. The cells were also decalcified with 0.6 M HCl at 4°C overnight. Free calcium in the supernatants was determined using a calcium colorimetric assay (Sigma, MAK022-1KT) according to the manufacturer's instructions. Cells were washed with PBS twice and harvested in lysis buffer (1 mM NaOH/0.1% SDS) for protein extraction. The total protein concentration was determined with bicinchoninic acid (BCA) protein assay kit (Thermo Fisher, 23235). Calcium content was normalized to total cell protein and expressed as μg/mg protein.

### 2.6 Intracellular zinc detection

hVICs were cultured with control or calcifying media in the presence of 20 μM $ZnSO_4$ and/or 20μM TPEN for up to 7 days. Intracellular zinc was detected using the zinc-selective indicator FluoZin-3 AM (Invitrogen, F24195) following the manufacturer's instructions. Briefly, cells were incubated with 1 μM of FluoZin-3 AM containing 0.02% Pluronic F-127 (Invitrogen, F24195) for 30 min at 37°C. After washing with $Ca^{2+}$ and $Mg^{2+}$ free PBS, cells were incubated for an additional 30 min at 37°C, and mounted in warm PBS buffer containing DAPI. Fluorescence images were detected by excitation at 488 nm and emission at 542 nm under a Leica DMRB fluorescence microscope (Leica SP8).

### 2.7 Immunofluorescence staining

To evaluate the expression of GPR39, hVICs were cultured with control or calcifying media in the presence or absence of 20 μM $ZnSO_4$ for up to 7 days. Cells were fixed, permeabilized with 0.3% triton x-100 (Beyotime Biotechnology, P0013B) and incubated overnight at 4°C with anti-GPR39 (1:500, Abcam, ab229648). After washing with PBS, cells were incubated with Alexa Fluor®488 anti-rabbit antibodies (1:1000, Invitrogen, A11008) in blocking buffer at 37 °C for 1 hr in the dark. Glass coverslips were then stained with DAPI

and fluorescence signal was detected under Leica DMRB fluorescence microscope (Leica SP8). Negative controls were carried out simultaneously by incubating with equivalent concentrations of normal rabbit IgG (Santa Cruz, sc2025) instead of primary antibody.

### 2.8 Quantitative RT-PCR

Total RNA was extracted from hVICs using TaKaRa MiniBEST Universal RNA Extraction Kit (Takara, 9767) and from human aortic valves using Trizol (Invitrogen, 1596026) according to the manufacturers' instructions. RNA was quantified and reverse transcribed using PrimeScript™ RT Master Mix (Takara, RR036A). Quantitative RT-PCR was performed with SYBR Premix Ex Taq II (Takara, RR820A) in the QuantStudio 5 real-time system (Life technologies). Each PCR was run in triplicate. All gene expression data were calculated using the $2^{-\Delta\Delta CT}$ method and normalized against GAPDH. The control values were expressed as 1 to indicate a precise fold change value for each gene of interest. The primer sequences for target genes are summarized in Supplementary material Table V.

### 2.9 Transfection of siRNAs

hVICs were seeded at the density of $1.0x10^5$ cells/well in 6-well plates and transfected with 25 nM *GPR39* siRNA (RIBOBIO), *ZIP13* siRNA (RIBOBIO), *ZIP14* siRNA (RIBOBIO), *ERK1/2* siRNA (RIBOBIO) or Non-targeting (siNC) siRNA (RIBOBIO) using Lipofectamine RNAiMAX (Invitrogen, 13778) following the manufacturer's instructions. The knockdown efficiency of siRNAs used in the present study was confirmed by quantitative RT-PCR and western blotting. For long-term hVIC *in vitro* calcification, cells were re-transfected at day 3 and incubated with calcifying media for up to 7 days. The siRNA sequences for gene silencing are summarized in Supplementary material Table VI.

### 2.10 Western blotting

hVICs were harvested with RIPA lysis buffer (Beyotime Biotechnology, P0013B) containing 1 mM protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (Beyotime Biotechnology, ST505). Western blotting was performed. Equal amounts of protein lysates were separated on SDS-Polyacrylamide gels and transferred to PVDF membranes. The membranes were incubated overnight at 4 °C with primary antibodies: anti-caspase 3 (1:2000, Cell Signaling Technology, 9662S), anti-cleaved caspase 3 (1:2000, Cell Signaling Technology, 9664S), anti-pAKT (1:4000, Cell Signaling, 4060S), anti-AKT (1:4000, Cell Signaling Technology, 9272), anti-pERK (1:4000, Cell Signaling Technology, 9101), anti-ERK (1:4000, Cell Signaling Technology, 4695), Anti-GPR39 (1:2000, Abcam, ab229648), Anti-RUNX2 (1:2000, Abcam, ab23981), Anti-MSX2 (1:2000, Santa Cruz, sc-393986), Anti-ZIP13 (1:2000, Abcam, ab106586), Anti-ZIP14 (1:2000, Abcam, ab106568), anti-β-actin (1:2000, Santa Cruz, J1116). Subsequently, membranes were incubated with HRP-conjugated anti-mouse (1:4000, Cell Signaling Technology, 7076S) or anti-rabbit (1:4000, Cell Signaling Technology, 7074S) secondary antibodies for 1 hr at room temperature. The immune complexes were visualized by chemiluminescence Lumi-Light Western Blotting Substrate (Millipore, WBKLS0500). Semi-quantitative assessment of band intensity was achieved by using ImageJ software (National Institutes of Health).

### 2.11 Cell death assay

hVICs were seeded at a density of $1.0x10^4$/wells in 96-well plates and incubated with control or calcifying media in the presence or absence of 20 μM $ZnSO_4$ for up to 7 days. Cell death was measured using Cytotoxicity lactic dehydrogenase (LDH) Assay Kit (Beyotime Biotechnology, C0038) following the manufacturer's instructions.

### 2.12 Calcium phosphate precipitation assay

Calcium phosphate precipitation assay was performed as previously described[15]. Briefly, the indicated concentrations of $ZnSO_4$ (0-20μM) was incubated with a homogeneous system containing 10 mM $CaCl_2$ (Sigma, 793639) and 10 mM sodium phosphate buffer (pH7.4, Sigma, 342483) in 500 mM HEPES buffer (pH7.4, Sigma, RDD002) for 10 min at room temperature. The samples were then centrifuged at 1890g for 30 sec and the obtained pellet was dissolved in 0.6 M HCl. Calcium content in the pellet was determined using a calcium colorimetric assay (Sigma, MAK022-1KT).

### 2.13 Apoptosis assay

Apoptotic hVICs were determined by manually counting pyknotic nuclei after staining with DAPI (Cell Signaling, 4083S). In addition, hVICs in different stages of apoptosis were analyzed by flow cytometry using Dead Cell Apoptosis Kit with Annexin V Alexa Fluor™ 488 & Propidium Iodide (PI) (Invitrogen, V13241) according to the manufacturer's instructions. In brief, hVICs were cultured with control or calcifying media in the presence or absence of 20 μM $ZnSO_4$ for 3 days. Cells were harvested by trypsinization, washed with cold PBS and stained with Annexin V Alexa Fluor™ 488 and PI. 30,000 cell events were recorded on a BD FACS Calibur (Becton, Dickinson & Company) and data were analyzed with FlowJo 8.8.4 flow cytometry analysis software (Tree Star Inc.).

### 2.14 Histology and immunohistochemistry

Human aortic valves tissues were fixed with 4% PFA for 24 hrs, dehydrated and embedded in paraffin wax before sectioning at 7 μm using standard procedures. For evaluation of calcium deposition, sections were de-waxed in xylene and stained with 2% Alizarin Red S solution for 5 min. After removal of the excess dye, sections were rinsed in 100% acetone and %50:%50 acetone-xylene. Sections were then cleared in 100% xylene for 5 min and mounted using neutral balsam (Solarbio, G8590). Light microscopy images were obtained by a scanning light microscope (Leica CS2).

For immunohistochemistry, sections were subjected to sodium citrate (pH 6.0) for antigen retrieval for 10 min at 95 °C. Endogenous peroxidase activity and non-specific antibody binding were blocked before overnight incubation at 4 °C with anti-GPR39 antibody (1:500, Abcam, ab229648). The sections were then washed in PBS, and incubated with secondary antibody using ChemMate™ EnVision™ Detection Kit (Gene Tech, GK500710) following the manufacturer's instructions. The sections were finally counterstained with haematoxylin and eosin, dehydrated, and mounted in neutral balsam (Solarbio, G8590). Control sections were incubated with equal concentrations of normal rabbit IgG (Santa Cruz, sc2025) in place of the primary antibody. Images were obtained with a digital whole slide

scanner (Leica Aperio CS2).

### 2.15 Statistical analysis

All experiments were repeated at least 3 times and the representative results are shown. All values are expressed as mean ±SEM. Statistical analysis was performed using GraphPad Prism 6 (La Jolla, CA) software. After confirming a normal distribution using the Shapiro-Wilk test, data were analysed using unpaired Student's t-test for comparison of two groups, one-way analysis of variance followed by Bonferroni post-test for comparison of multiple groups or a suitable non-parametric test such as Mann–Whitney. $P < 0.05$ was considered to be statistically significant.

## 3. Results

### 3.1 Zinc supplementation inhibits hVIC *in vitro* calcification and osteogenic differentiation

Initial studies were performed to validate the hVIC *in vitro* calcification model. In accordance with our previous report[8], hVICs showed positive staining for both SM22-α and Vimentin (Supplementary Figure II A). As revealed by alizarin red staining and quantitative calcium analysis, hVICs cultured under calcifying media showed significantly increased calcium deposition (12.15 fold, $p<0.001$; Supplementary Figure II B and C) at day 7. The osteogenic related genes including alkaline phosphatase (*ALPL*) (3.8 fold, $p<0.001$), Msh Homeobox 2 (*MSX2*) (3.9 fold, $p<0.01$), and *RUNX2* (1.6 fold, $p<0.001$) were dramatically up-regulated in calcified hVICs compared to non-calcified hVICs (Supplementary Figure II D). Furthermore, increased cell death (11.5% at Day7, $p<0.001$) and apoptosis (2.0 fold, $p<0.05$) were observed during the process of hVIC *in vitro* calcification (Supplementary Figure II E and F). These results are consistent with previous reports[4, 8].

To evaluate the possible effects of zinc on hVIC *in vitro* calcification, hVICs were treated with 5-20 μM $ZnSO_4$ in the presence of calcifying media for up to 7 days. Alizarin red staining and quantitative calcium analysis showed that 20μM $ZnSO_4$ significantly attenuated calcium deposition in hVICs at day 7 (53% decrease, $p<0.001$; Figure 1A and 1B). To determine whether the inhibitory effect of $ZnSO_4$ on hVICs *in vitro* calcification was specifically due to zinc ions, a specific zinc chelator TPEN was used. FluoZin-3 (a zinc-selective indicator) staining revealed that 20 μM TPEN dramatically abolished zinc accumulation in hVICs treated with 20 μM $ZnSO_4$ at day 3 and day 7 (Figure 1C). As expected, 20 μM TPEN significantly blunted the inhibitory effect of 20 μM $ZnSO_4$ on hVIC *in vitro* calcification ($p<0.001$; Figure 1D). Furthermore, $ZnCl_2$ treatment also significantly inhibited calcium deposition in hVICs at day 7 (39% decrease, $p<0.001$; Figure 1E). Considering the important role of osteogenic differentiation of hVICs in aortic valve calcification[4, 8], we next assessed whether zinc supplementation could reduce osteogenic related gene expression in hVICs. The up-regulation of *ALPL* and *MSX2* in hVICs induced by calcifying media was significantly attenuated by zinc treatment after 7 days (Figure 1F and 1G). It has been reported that serum albumin is the major zinc carrier in blood and responsible for its system distribution[18]. We showed that 1mg/ml albumin did not alter the inhibitory

effect of zinc on hVIC calcification (Figure 1H). Taken together, these data suggest that zinc supplementation specifically inhibits hVIC *in vitro* calcification.

### 3.2 Zinc prevents apoptosis during hVIC *in vitro* calcification

Apoptosis has been previously reported to play a crucial role in initiation and progression of aortic valve calcification[9-11], we therefore undertook a detailed assessment of apoptosis in hVICs following zinc treatment. 20 μM $ZnSO_4$ treatment significantly reduced cell death ($p<0.05$; Figure 2A), apoptotic nuclei ($p<0.001$; Figure 2B) and apoptosis (50% decrease, $p<0.05$; Figure 2C) in hVICs cultured with calcifying media. Western blotting also showed that zinc supplementation prevented up-regulation of cleaved caspase3 in hVICs cultured with calcifying media at day 7 (Figure 2D, $p<0.05$). Calcium phosphate precipitation was not affected by $ZnSO_4$ treatment (Figure 2E). In addition, zinc supplementation did not alter calcium phosphate deposition on fixed hVICs (Supplementary Figure III A and B). These data suggest no direct physicochemical inhibition of calcium/phosphate deposition by $ZnSO_4$. We further examined the expression of TNFα and TFGβ, which are the important cytokines that involved in calcification through regulation of apoptosis[9, 10]. Zinc supplementation significantly attenuated the up-regulation of *TNFα* mRNA expression during hVIC *in vitro* calcification (45.2% decrease, Figure 2F, $p<0.01$). However, *TGFβ* mRNA expression was not altered in hVICs cultured with calcifying media in the absence or presence of zinc supplementation (Figure 2G). Taken together, our data suggests that zinc supplementation reduces hVIC *in vitro* calcification at least in part through inhibition of apoptosis.

### 3.3 Zinc inhibits hVIC *in vitro* calcification and apoptosis through activation of the ERK1/2 signaling pathway

To elucidate the underlying mechanisms of the protective effect of zinc on hVIC calcification, we investigated the activation of AKT and EKR1/2, which have been previously reported as key regulators of hVIC calcification[19, 20]. 20 μM $ZnSO_4$ treatment significantly induced phosphorylation of ERK1/2 after 10 min ($p<0.01$) and 30 min ($p<0.01$), but not after 60 min, while AKT phosphorylation was not induced by addition of $ZnSO_4$ (Figure 3A). 20 μM $ZnSO_4$ treatment effectively inhibited hVIC *in vitro* calcification, as determined by alizarin red staining (Figure 3B) and calcium quantitative analysis ($p<0.001$, Figure 3C). Accordingly, the inhibitory effect of $ZnSO_4$ on hVIC calcification was significantly abolished in the presence of the ERK1/2 inhibitor PD98059 ($p<0.01$, Figure 3B and 3C). ERK1/2 inhibitor PD98059 by itself had no effect on hVIC calcification (Supplementary Figure IV A and B). Moreover, the inhibition of $ZnSO_4$ on cleaved caspase3 (as an indication of apoptosis) expression ($p<0.01$, Figure 3D) and cell death ($p<0.01$, Figure 3E) in hVICs was also prevented by PD98059. In addition, siRNA-mediated knockdown of ERK1/2 also blunted the inhibitory effect of zinc on hVIC *in vitro* calcification ($p<0.05$, Figure 3F). ERK1/2 siRNA knockdown efficiency was confirmed by quantitative RT-PCR and western blotting (Supplementary Figure V A-C). These data support that activation of the ERK1/2 signaling pathway plays a key role in mediating the protective effect of zinc on hVIC calcification and apoptosis.

### 3.4 Zinc signals through the GPR39 receptor to activate ERK1/2 signaling pathway and

**inhibit hVIC calcification**

The zinc-sensing receptor GPR39 plays a key role in mediating zinc intracellular signaling pathways[21]. Additional experiments were therefore performed to examine the role of GPR39 in zinc mediated inhibition of hVIC calcification. The expression of GPR39 was detected in hVICs isolated from 5 patients with CAVD (Figure 4A). The endogenous expression of GPR39 was suppressed by transfection of GPR39 siRNA. Western blotting analysis showed that GPR39 siRNA 2 resulted in a significant reduction of GPR39 protein expression (Figure 4B). As expected, silencing of GPR39 attenuated zinc induced phosphorylation of ERK1/2 (20% decrease, $p<0.01$; Figure 4C). In addition, silencing of GPR39 by itself did not alter hVIC *in vitro* calcification, but significantly blunted the inhibitory effect of zinc supplementation on hVIC *in vitro* calcification ($p<0.05$; Figure 4D and E). siRNA knockdown of GPR39 also attenuated the inhibitory effects of zinc on hVIC apoptosis ($p<0.05$; Figure 4F) and the osteogenic gene *MSX2* ($p<0.05$; Figure 4G) and *BMP2* mRNA expression ($p<0.001$; Figure 4H). The inhibitory effect of zinc on *RUNX2* mRNA expression was not significantly affected by knockdown of GPR39 (Figure 4I). Our data support a key role of GPR39 in mediating the anti-calcific effects of zinc supplementation on hVICs.

### 3.5 Knockdown of *ZIP13* or *ZIP14* inhibits hVIC *in vitro* calcification

The human zinc transporter ZIP family consisting of 14 members functions to increase the cytosolic zinc accumulation by transporting zinc into the cytosol from the extracellular space or from intracellular compartments[22]. Among the 14 members of ZIP family examined, only *ZIP13* (5.0 fold, $p<0.05$) and *ZIP14* (1.8 fold, $p<0.05$) mRNA expression were significantly induced in hVICs treated with 20 μM $ZnSO_4$ (Figure 5A). We therefore undertook further analysis to investigate the functional role of *ZIP13* and *ZIP14* in hVIC calcification. Knockdown efficiency of *ZIP13* and *ZIP14* was confirmed by quantitative RT-PCR and western blotting (Supplementary Figure V D-I). Knockdown of *ZIP13* resulted in a significant decrease of calcium deposition in hVICs (30% decrease, $p<0.05$, Figure 5B and 5C). This inhibitory effect was further exacerbated in the presence of 20 μM $ZnSO_4$ (50% decrease, $p<0.05$, Figure 5B and 5C). Consistent with these data, knockdown of *ZIP13* significantly reduced mRNA expression of the osteogenic related gene *MSX2* (51.1% decrease, $p<0.001$, Figure 5F) in hVICs cultured with calcifying media after 7 days. However, this inhibition was abolished by zinc supplementation (Figure 5F). Knockdown of *ZIP13* also decreased the osteogenic gene *ALPL* mRNA expression in hVICs cultured with calcifying media in the absence (63.6% decrease, $p<0.05$, Figure 5G) or presence (70.8% decrease, $p<0.05$, Figure 5G) of 20 μM $ZnSO_4$ after 7 days. Knockdown of *ZIP14* attenuated hVIC *in vitro* calcification (19% decrease, $p<0.05$, Figure 5D and 5E), but the addition of zinc to the knockdown of ZIP14 did not provide further reduction of the calcification process in hVIC cultures (Figure 5E). In addition, knockdown of *ZIP14* resulted in a significant reduction in *MSX2* mRNA expression in hVICs cultured with calcifying media in the absence (51.1% decrease, $p<0.001$, Figure 5F) or presence (43.5% decrease, $p<0.01$, Figure 5F) of 20 μM $ZnSO_4$ after 7 days. However, knockdown of *ZIP14* did not alter *ALPL* mRNA expression in hVICs (Figure 5G). We did not see any significant difference in the expression of ZIP13 and ZIP14 between un-calcified and calcified human aortic valves (Supplementary Figure VI A

and B). Additionally, we observed that either knockdown of ZIP13 or ZIP14 dramatically reduced the cytosolic zinc concentrations in hVICs in the absence or presence of zinc supplementation (Figure 5H and 5I). Taken together, our data suggest an important role of zinc transporter *ZIP13* and *ZIP14* in hVIC calcification and osteogenic differentiation.

### 3.6 GPR39 expression is decreased in calcified human aortic valves

To better understand the role of GPR39 in CAVD, expression of GPR39 was assessed in 4 non-calcified and 4 calcified human aortic valves. Clinical characteristics of these patients are shown in Supplementary material Table II. Calcium deposition in human aortic valves was confirmed by positive staining of alizarin red (Figure 6A). Immunohistochemistry showed that GPR39 expression was dramatically decreased in calcified aortic valves compared to non-calcified aortic valves (Figure 6A). This observation was further confirmed by a western blotting analysis of 4 non-calcified aortic valves and 4 calcified aortic valves, which showed a significant reduction of GPR39 expression in calcified human aortic valves (30% decrease, $p<0.05$; Figure 6B). These data are the first to show that GPR39 expression is decreased in calcified human aortic valves. Furthermore, zinc serum levels were also significantly decreased in patients with CAVD compared to healthy volunteers ($p<0.01$; Figure 6C). These patients had aortic valve sclerosis, which is an early stage of CAVD and characterized by thickening and calcification of the aortic valve without obstruction of ventricular outflow (Supplementary Figure I and Supplementary material Table III). There was no significant difference in age and sex between healthy volunteers and CAVD patients. Body Mass Index (BMI) was significantly decreased in CAVD patients compared to healthy volunteers ($p<0.05$, Supplementary material Table III). A forward stepwise multivariate logistic analysis (likelihood ratio) including zinc and BMI was performed. Increasing zinc serum levels was shown to be a significant protective factor of CAVD after adjusted the BMI (OR=0.135; C.I.: 0.022~0.815; P=0.029). The statistical analysis are provided as Supplementary Methods.

### 3.7 Reduction of GPR39 in calcified hVICs is blunted by zinc supplementation

In agreement with our previous observations (Figure 6A and B), our *in vitro* studies showed that GPR39 expression was also decreased at day 7 in calcified hVICs compared to non-calcified hVICs, as demonstrated by immunofluorescence staining ($p<0.001$; Figure 7A) and western blotting ($p<0.01$; Figure 7B). Interestingly, zinc supplementation significantly attenuated the reduction of GPR39 in calcified hVICs (Figure 7A and 7B).

## 4. Discussion

The current study identifies that zinc as a novel inhibitor of CAVD. Zinc supplementation significantly inhibits hVIC *in vitro* calcification, and this inhibitory effect is abolished by the zinc chelator TPEN. Mechanistically, zinc supplementation inhibits osteogenic differentiation and prevents apoptosis of hVICs, which is at least in part mediated by GPR39-dependent ERK1/2 signaling pathway. Also, a significant reduction in the zinc sensing receptor GPR39 expression in human calcified aortic valves is observed, and zinc serum levels are decreased in patients with CAVD compared to healthy volunteers. Furthermore, either knockdown of zinc transporter ZIP13 or ZIP14 reduced hVIC *in vitro*

calcification and osteogenic differentiation. This study provides direct evidence to show that zinc supplementation inhibits the pathological process of CAVD, and highlights an important role of ZIP13 and ZIP14 in the progression of CAVD.

Zinc, a vital trace element for normal health, is associated with a number of human diseases, including cardiovascular disease, and diabetes. To our knowledge, the current study is the first report indicating that zinc supplementation effectively inhibits hVIC *in vitro* calcification. Zinc bioavailability is influenced by many factors in healthy individuals, including zinc status of individuals, total zinc concentration and availability of soluble zinc in the diet. The optimal serum zinc concentrations in adults are maintained between 13.8–22.9 μM[23]. We showed that 20 μM $ZnSO_4$ within this normal range was sufficient to inhibit calcium deposition in hVICs. In addition, zinc is predominantly carried by serum albumin in the blood[18]. Addition of serum albumin did not alter the inhibitory effect of zinc on hVIC *in vitro* calcification. Our further analysis using TPEN chelator and $ZnCl_2$ confirmed the specific inhibitory effect of zinc on calcification. Taken together, these data suggest that zinc is a key inhibitor of CAVD.

Previous studies have shown that osteogenic transition of hVICs plays a crucial role in the pathogenesis of CAVD[4, 8]. In the present study, zinc treatment significantly inhibited the osteogenic gene *MSX2* and *ALPL* mRNA expression after 7 days. These data are consistent with a recent study showing that zinc inhibits high phosphate-induced osteogenic transition of human VSMCs[15]. However, zinc has previously been reported to enhance osteogenic differentiation of human mesenchymal stem cells via up-regulation of RUNX2[24]. The existence of cell-mediated differences may account for the different osteogenic responses among these cells following zinc treatment.

Apoptosis is a key regulator of initiation and progression of CAVD[9-11]. Apoptotic bodies expose phosphatidylserine on the outer membranes and generate a potential calcium-binding site suitable for hydroxyapatite deposition[25, 26]. We showed that zinc attenuated apoptosis during hVIC *in vitro* calcification process, but did not affect calcium phosphate precipitation. Therefore, zinc-mediated inhibitory effect on hVIC calcification may involve inhibition of apoptosis. These results also support previous reports demonstrating that zinc supplementation prevents apoptosis in a number cell types including cardiomyocytes[27], VSMCs[28], and in cardiac allografts[29]. In the present study, we showed that zinc supplementation significantly attenuated *TNFα* mRNA expression during hVIC *in vitro* calcification. TNFα, a pleiotropic cytokine, regulates a range of cellular activities including proliferation, differentiation and apoptosis. TNFα-mediated signaling pathways have been shown to play an important role in cardiovascular calcification[30, 31]. Furthermore, previous studies have reported TNFα accelerates the calcification of hVICs through the BMP2-Dlx5 pathway[32]. Future studies are required to address whether the inhibitory effect of zinc on hVIC calcification is mediated through down-regulation of TNFα or the reduced expression of TNFα is just a consequence of cellular remodeling in calcification.

The human ZIP family functions to increase cytoplasmic zinc concentrations[22]. We

revealed that ZIP13 and ZIP14 were the most up-regulated ZIP family members in hVICs following zinc treatment. ZIP13 is localized in intracellular vesicles and releases zinc from vesicular stores[33]. We showed that knockdown of ZIP13 attenuated hVIC *in vitro* calcification, and this inhibitory effect was further increased in the presence of zinc treatment. These results suggest a zinc storage in intracellular vesicles plays a key role in hVIC *in vitro* calcification. Interestingly, it has been reported that *Zip13* knockout mice show reduced osteogenesis and abnormal cartilage development, which is mediated at least in part through TGF-β/bone morphogenetic protein (BMP) signaling pathways[33]. In addition, ZIP13 has been shown to suppress beige adipocyte biogenesis and energy expenditure by regulating c/ebp-beta expression[34]. Consistent with this, knockdown of ZIP13 reduced osteogenic gene *MSX2* and *ALPL* mRNA expression in hVICs. ZIP14 is responsible for zinc uptake by cells[35], and has been reported to be a critical regulator of glucose homeostasis and beta-cell function[35, 36]. We found that knockdown of ZIP14 in hVICs attenuated calcium deposition. However, this inhibitory effect was abolished by zinc supplementation. This is likely due to a compensatory role of other zinc transporters in uptake of zinc into cytoplasm from extracellular space when additional zinc supplementation exists. Additionally, knockdown of ZIP14 decreased *MSX2* mRNA expression in hVICs, suggesting ZIP14-mediated zinc uptake may regulate osteogenic differentiation of hVICs. Taken together, our data expand upon these findings and highlight the important role of ZIP13 and ZIP14 in the maintenance of intracellular zinc homeostasis in hVIC *in vitro* calcification and their potential roles in CAVD. The mechanisms underlying the anti-calcific effects of ZIP13 or ZIP14 silencing are currently elusive and require further study.

It should be noted that extracellular zinc and intracellular zinc have opposite role in the regulation of hVIC calcification and osteogenic differentiation. Extracellular zinc may signal through GPR39 to activate a number of intracellular signaling pathways[21], thereby regulating hVIC calcification and osteogenic differentiation. However, intracellular zinc mediated by zinc transporters may act as co-factors for a number of enzymes such as ALPL[37], which play an important role in hVIC calcification and osteogenic differentiation.

In order to characterize the underlying mechanisms through which zinc exerts its protective on hVICs, the PI3-kinase/AKT and MAPK/ERK1/2 signaling pathways were investigated. These pathways play an important role in a wide range of cellular functions, including cell proliferation, cell survival, and calcification[38, 39]. We showed that zinc treatment only induced the phosphorylation of ERK1/2 in hVICs, which is consistent with previous reports showing that activation of this pathway by zinc in skeletal muscle cells[40], myogenic cells[41], and colonocytes[42]. In contrast to these studies, no induction of AKT phosphorylation by zinc was observed in hVICs. We also demonstrated that either pharmacological or siRNA-mediated inhibition of ERK1/2 signaling pathway significantly blunted the protective effect of zinc on hVIC *in vitro* calcification and apoptosis. Indeed, the ERK1/2 signaling pathway has been shown to regulate calcification in osteoblasts[43], VSMCs[44], and human VICs[45]. However, some previous studies have reported that the activation of ERK1/2 signaling pathway exerts pro-calcific effects in valvular calcification. The disparate findings between our studies and theirs may result from different pro-calcific environments (that is

high calcium and phosphate vs. fibrin and tissue culture polystyrene) and species (human vs. porcine)[19]. In addition, inhibition of ERK1/2 signaling pathway has been shown to attenuate aortic valve disease processes in an Emilin1-deficient mouse model[46]. The abnormalities of aortic valves in these mice include early elastic fiber fragmentation and aberrant angiogenesis, however no aortic valve calcification was detected at any stage[46, 47]. Further investigations are required to examine the effects of zinc-mediated ERK1/2 signaling pathway on aortic valve calcification using mouse models of CAVD. Taken together, our data confirms and extends these previous reports indicating the importance of the ERK1/2 signaling pathway in the regulation of CAVD.

GPR39 has been functionally characterized as a zinc-dependent, G-protein coupled receptor that senses changes in extracellular zinc and mediates zinc dependent cellular signaling pathways[48]. It is widely expressed by a number of cell types including vascular endothelial cells and VSMCs[15, 21]. We extended on these previous studies showing that GPR39 is expressed by hVICs, and silencing of GPR39 abrogated zinc activation of ERK1/2 signaling pathway. We also showed that inhibition of ERK signaling pathway attenuated zinc-mediated inhibitory effect on hVIC apoptosis and calcification. In addition, knockdown of GPR39 blunted the inhibitory effect of zinc supplementation on hVIC *in vitro* calcification, osteogenic differentiation and apoptosis. Taken together, these data support that zinc signals through GPR39 to activate ERK1/2 signaling pathway, thereby suppressing osteogenic differentiation and apoptosis, which could inhibit hVIC calcification.

The most important observation in this study was that the zinc sensing receptor GPR39 expression was decreased in calcified hVICs and human aortic valves from patients with CAVD. The pathological role of GPR39 deficiency has been previously described. Deletion of GPR39 in mice results in zinc deficiency symptoms including depression, accelerated gastric emptying, and increased fecal excretion[49]. Furthermore, GPR39 knockout mice display enhanced high fat-induced obesity due to altered adipocyte metabolism[50]. Interestingly, we observed that zinc treatment prevented the reduction of GPR39 in calcified hVICs. This is in consistent with a previous report showing that zinc deficiency reduces expression of the GPR39 receptor in the mouse frontal cortex[51]. These data raise the possibility that zinc supplementation may reverse aortic valve calcification. It would be interesting to investigate whether zinc supplementation could reverse the established aortic valve calcification in mouse models of CAVD. Nonetheless, our data provide novel evidence that support a key role of GPR39 deficiency in the pathogenesis of CAVD.

Reduced zinc levels are commonly seen in patients with chronic kidney disease (CKD)[52]. In addition, reduced zinc serum levels are also associated with carotid artery atherosclerosis in hemodialysis patients[53]. Interestingly, a recent study has shown that zinc serum concentrations inversely correlated with serum calcification propensity in CKD patients[15]. In accordance with these studies, we observed a significant reduction in zinc serum levels in a cohort of Chinese CAVD patients compared to healthy volunteers. These data together with our hVIC *in vitro* calcification studies highlight the important inhibitory role of zinc in the development of CAVD.

It should be noted that this study may contain some limitations. The hVICs used in the present study were isolated from diseased aortic valve tissues, which may be more sensitive to apoptosis and cell death. In addition, the inhibitory or reversal effect of zinc on CAVD was not tested *in vivo*. Future studies are required to investigate whether zinc supplementation can inhibit or reverse aortic valve calcification using mouse models of CAVD.

In conclusion, we report that zinc as a novel inhibitor of CAVD. The zinc sensing receptor GPR39 is reduced in calcified aortic valves from patients with CAVD. The anti-calcific effect of zinc on hVIC calcification is at least in part mediated through inhibition of apoptosis and osteogenic differentiation via GPR39 dependent EKR1/2 signaling pathway. Our study also highlights an important role of zinc transporter ZIP13 and ZIP14 in CAVD. This work warrants further investigations of zinc supplementation or zinc transporter ZIP13 and ZIP14 as a potential novel therapeutic strategy for treatment of CAVD.

## Supplementary material

Supplementary material is available at Cardiovascular Research online.

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## Conflict of interest

None.

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## Figure legends

**Figure 1. Zinc inhibits hVICs *in vitro* calcification and osteogenic differentiation.** hVICs were exposed to control (Ctr), calcification media (Cal), $ZnSO_4$ with or without TPEN, $ZnCl_2$, and $ZnSO_4$ with or without albumin for up to 7 days as indicated. A. Representative alizarin red S staining images at day 7 (n=3). Plate view (upper) and microscopic view (lower), scale bar 100 μm. B. Quantitative calcium assay showed that $ZnSO_4$ inhibited hVIC *in vitro* calcification at day 7 (n=4). C. Representative confocal images of FluoZin-3 staining at day 3 and day 7 (n=3). Scale bar 50 μm. D. Quantitative calcium assay showed that 10 μM TPEN blunted the inhibitory effect of $ZnSO_4$ on hVIC *in vitro* calcification at day 7 (n=4). E. Quantitative calcium assay at day 7 (n=4). F and G. Quantitative RT-PCR for *MSX2* and *ALPL* mRNA expression in hVICs cultured with calcifying media after 7 days (n=6). H. Quantitative calcium assay showed that albumin did not affect the inhibitory effect of $ZnSO_4$ on hVIC *in vitro* calcification (n=4). Results are presented as mean ± SEM. ANOVA by Bonferroni post-test, *p < 0.05, ***p < 0.001 compared to control, $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p < 0.001 compared to calcification. $^{+++}$p < 0.001 compared to calcification with zinc treatment.

**Figure 2. Zinc prevents apoptosis of hVICs under calcifying conditions.** hVICs were exposed to control (Ctr) or calcification media (Cal) with or without 20 μM of $ZnSO_4$ treatment. A. Cell death at day 3 and day 7 (n=6). B. Representative apoptotic nuclei images of DAPI staining (left panel) and quantitative analysis of the percentage of apoptotic cells (right panel). White arrows indicate apoptotic cells, scale bar 10 μm (n=11). C. Flow cytometry analysis of Annexin V and Propidium Iodide (PI) staining (upper panels) and quantification of the percentage of early apoptotic cells (Q3, lower panel) at day 3 (n=3). D. Representative western blot of Caspase 3, cleaved Caspase 3, and β-actin (n=4). E. Calcium phosphate precipitation analysis showed that $ZnSO_4$ did not affect calcium/phosphate deposition (n=4). F. Quantitative RT-PCR for *TNFα* mRNA expression in hVICs cultured with calcifying media after 7 days (n=6). G. Quantitative RT-PCR for *TGFβ* mRNA expression (n=5 or 6). Results are presented as mean ± SEM. ANOVA by Bonferroni post-test. *p < 0.05, **p < 0.01, ***p < 0.001 compared to control. $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, $^{\#\#\#}$p < 0.001 compared to calcification.

**Figure 3. Zinc inhibits hVICs calcification and apoptosis through activation of the ERK1/2 signaling pathway.** hVICs were exposed to control (Ctr), calcification media (Cal), 20 μM $ZnSO_4$ treatment with or without 10 μM ERK inhibitor (PD98059) or siERK1/2 as indicated. A. hVICs were exposed to $ZnSO_4$ for 10, 30, and 60 minutes. Representative western blot of phospho-AKT (p-AKT), total AKT, phospho-ERK (p-ERK), total ERK, and β-actin (left panel) and quantification of the relative protein expression (right panel) (n=4). B. Representative alizarin red S staining images at day 7 (n=3). Plate view (upper) and microscopic view (lower), scale bar 100 μm. C. Quantitative calcium assay at day 7 (n=4). D. Representative Western blot of Caspase 3, cleaved Caspase 3, and β-actin (n=4). E. Cell death at day 7 (n=6). F. Quantitative calcium assay at Day7 (n=4). Results are presented as mean ± SEM. ANOVA by Bonferroni post-test. *p < 0.05, **p < 0.01, ***p < 0.001 compared to control. $^{\#}$p < 0.05, $^{\#\#\#}$p < 0.001 compared to calcification, +p < 0.05, ++p < 0.01 compared to

calcification with zinc treatment.

**Figure 4. Zinc signals through the zinc-sensing receptor GRR39 to inhibit hVICs calcification.** hVICs were transfected with GPR39 siRNA (siGPR39) or negative control siRNA (siNC) and exposed to control (Ctr), calcification media (Cal) with or without 20 μM of $ZnSO_4$ treatment. A. Representative western blot of GPR39 from 5 independent hVICs isolation. B. GPR39 silencing efficiency using three different siRNA (n=3). GPR39 siRNA 2 was selected for subsequent experiments. C. Representative western blot of phospho-AKT (p-AKT), total AKT, phospho-ERK (p-ERK), total ERK, and β-actin after 30 minutes with $ZnSO_4$ (n=4). D. Representative alizarin red S staining images at day 7 (n=3). Plate view (upper) and microscopic view (lower), scale bar 100 μm. E. Quantitative analysis of calcium content at day 7 (n=5). F. Representative western blot of Caspase 3, cleaved Caspase 3, and β-actin (n=4). G-I. Relative mRNA expression of *MSX2*, *BMP2* and *RUNX2* (n=6). Results are presented as mean ± SEM. ANOVA by Bonferroni post-test. *p < 0.05, **p < 0.01, ***p < 0.001 compared to control plus siNC. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to calcification plus siNC. $^{+}p < 0.05$, $^{+++}p < 0.001$ compared to calcification with zinc treatment plus siNC.

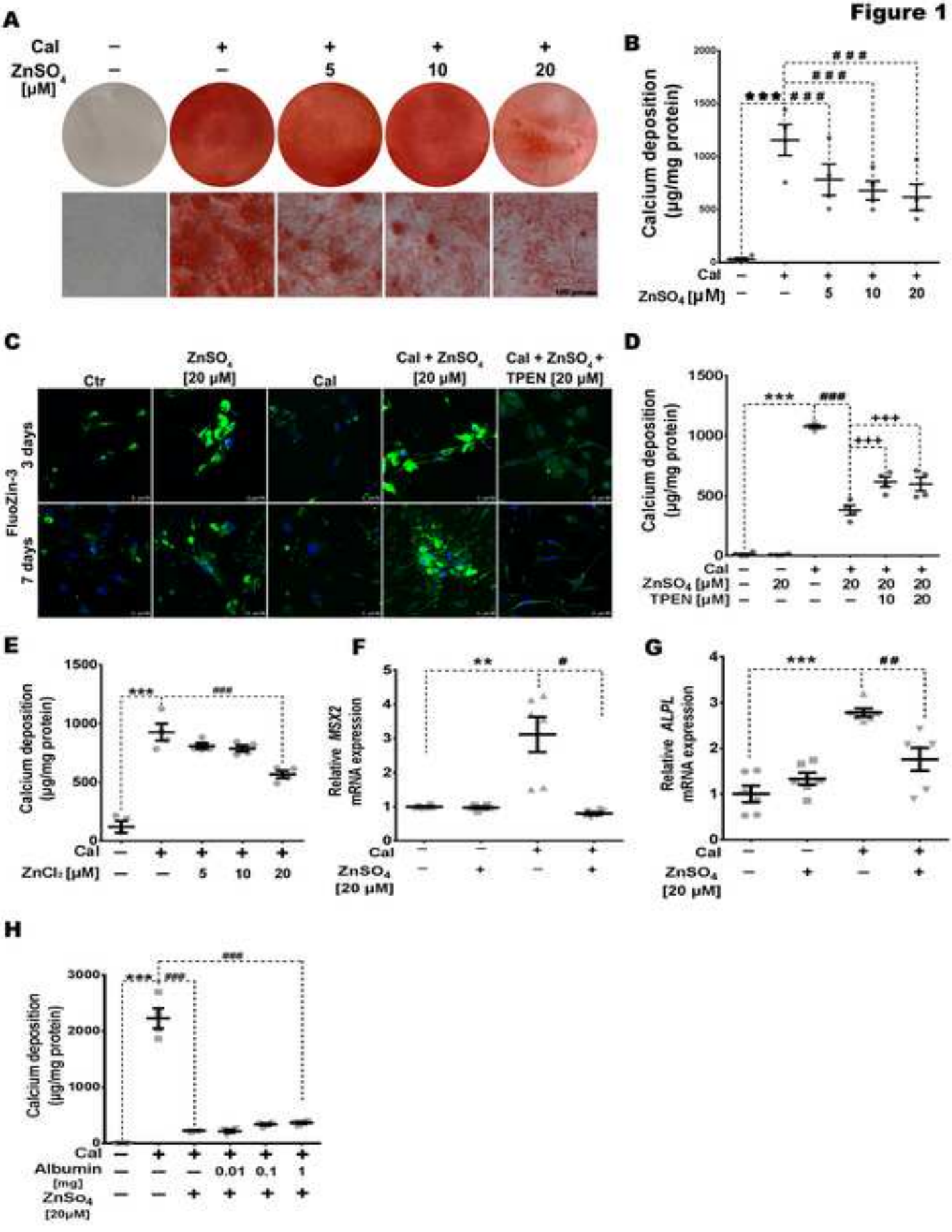
**Figure 5. Knockdown of ZIP13 or ZIP14 inhibits hVIC *in vitro* calcification and osteogenic differentiation.** hVICs were exposed to control (Ctr), or 20 μM $ZnSO_4$ treatment for 2 days, or transfected with siZIP13 or siZIP14, and exposed to calcification media (Cal) or 20 μM ZnSO4 treatment for up to 7 days as indicated. A. Relative mRNA expression of zinc transporter ZIP1-14 was examined by quantitative RT-PCR (n=6 or 8), unpaired Student's t-test. B. Representative alizarin red S staining images for hVICs transfected with siZIP13 at day 7 (n=3). C. Quantitative analysis of calcium content for hVICs transfected with siZIP13 at day 7 (n=5), ANOVA by Bonferroni post-test. D. Representative alizarin red S staining images for hVICs transfected with siZIP14 at day 7 (n=3). E. Quantitative analysis of calcium content for hVICs transfected with siZIP14 at day 7 (n=5), ANOVA by Bonferroni post-test. F-G. Relative mRNA expression of *MSX2* and *BMP2* at Day 7 (n=8). ANOVA by Bonferroni post-test. H-I. Representative confocal images of FluoZin-3 staining at day 7 and quantitative analysis of zinc fluorescence intensity (n=6), ANOVA by Bonferroni post-test. Scale bar 50 μm. Results are presented as mean ± SEM. *p<0.05, **<0.01, ***p < 0.001 compared to control. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared to calcification plus siNC. $^{+}p<0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ compared to calcification with zinc treatment plus siNC.

**Figure 6. GPR39 expression is down-regulated in calcified human aortic valves.** A. Immunohistologic evaluation of GPR39 expression in non-calcified and calcified human aortic valves (n=3). B. Representative western blot of GPR39 expression from 4 non-calcified or calcified human aortic valves (upper panels) and relative GPR39 expression (lower panel) (n=4), unpaired Student's t-test. C. Serum zinc concentrations in healthy and CAVD patients (n=15), Mann-Whitney test. Results are presented as mean ± SEM. *p < 0.05, **p < 0.01 compared to control.

**Figure 7. Zinc treatment prevents the down-regulation of GPR39 induced by**

**calcification *in vitro*.** hVICs were exposed to control (Ctr) or calcification media (Cal) with or without additional 20 µM of $ZnSO_4$ at day 3 and day 7. A. Representative confocal images of GPR39 immunostaining and relative GPR39 fluorescence intensity (lower panel) (n=8), ANOVA by Bonferroni post-test. B. Western blot of GPR39 expression (upper panel) and relative GPR39 expression (lower panel) (n=4), ANOVA by Bonferroni post-test. Results are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, **$p < 0.001$ compared to control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to calcification.

Figure 1


A
Cal
ZnSO4 [μM]
B
Calcium deposition (μg/mg protein)
ZnSO4 [μM]
C
Ctr
ZnSO4 [20 μM]
Cal
Cal + ZnSO4 [20 μM]
Cal + ZnSO4 + TPEN [20 μM]
FluoZin-3
3 days
7 days
D
Calcium deposition (μg/mg protein)
ZnSO4 [μM]
TPEN [μM]
E
Calcium deposition (μg/mg protein)
ZnCl2 [μM]
F
Relative MSX2 mRNA expression
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Relative ALPL mRNA expression
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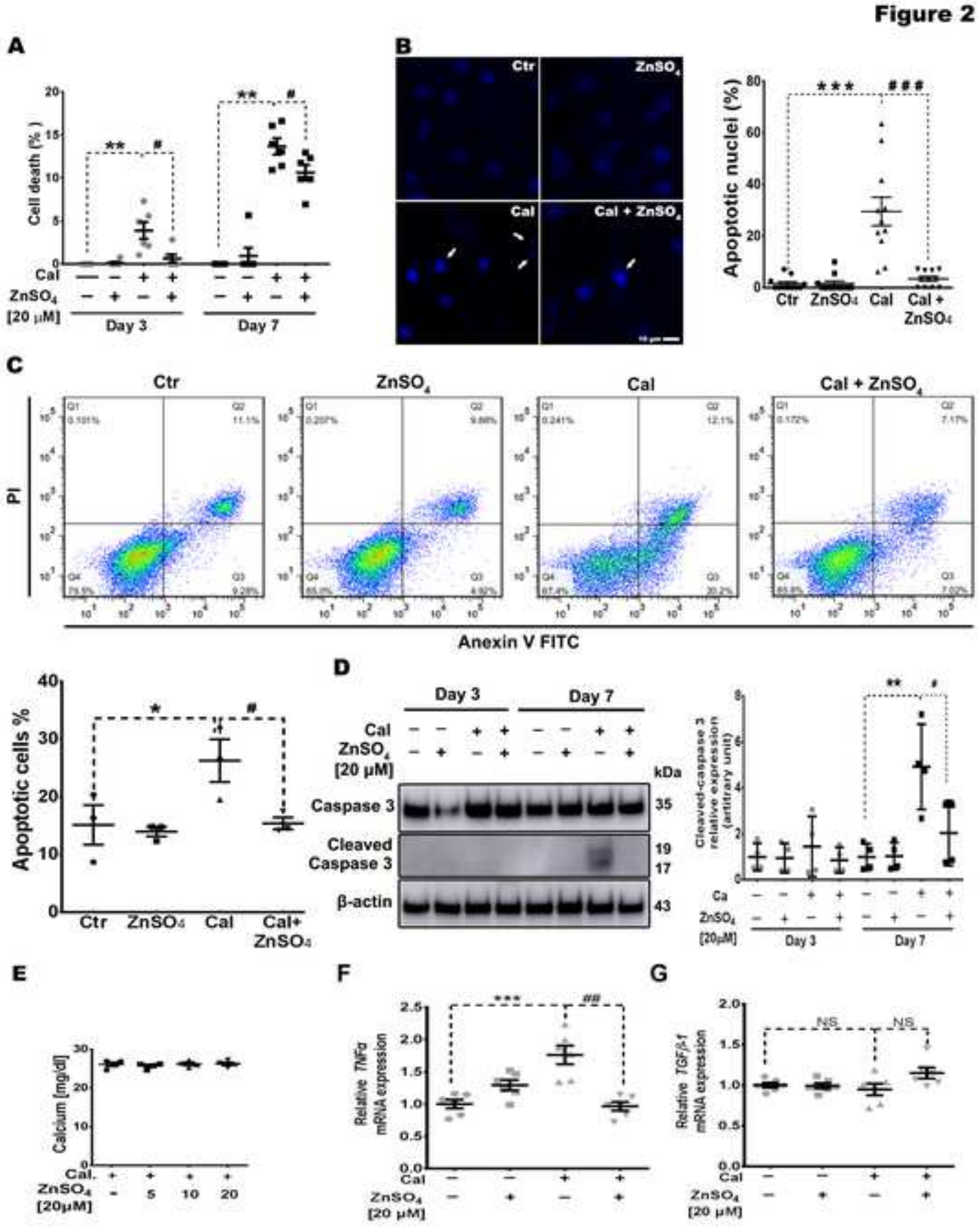
Albumin [mg]
ZnSo4 [20μM]



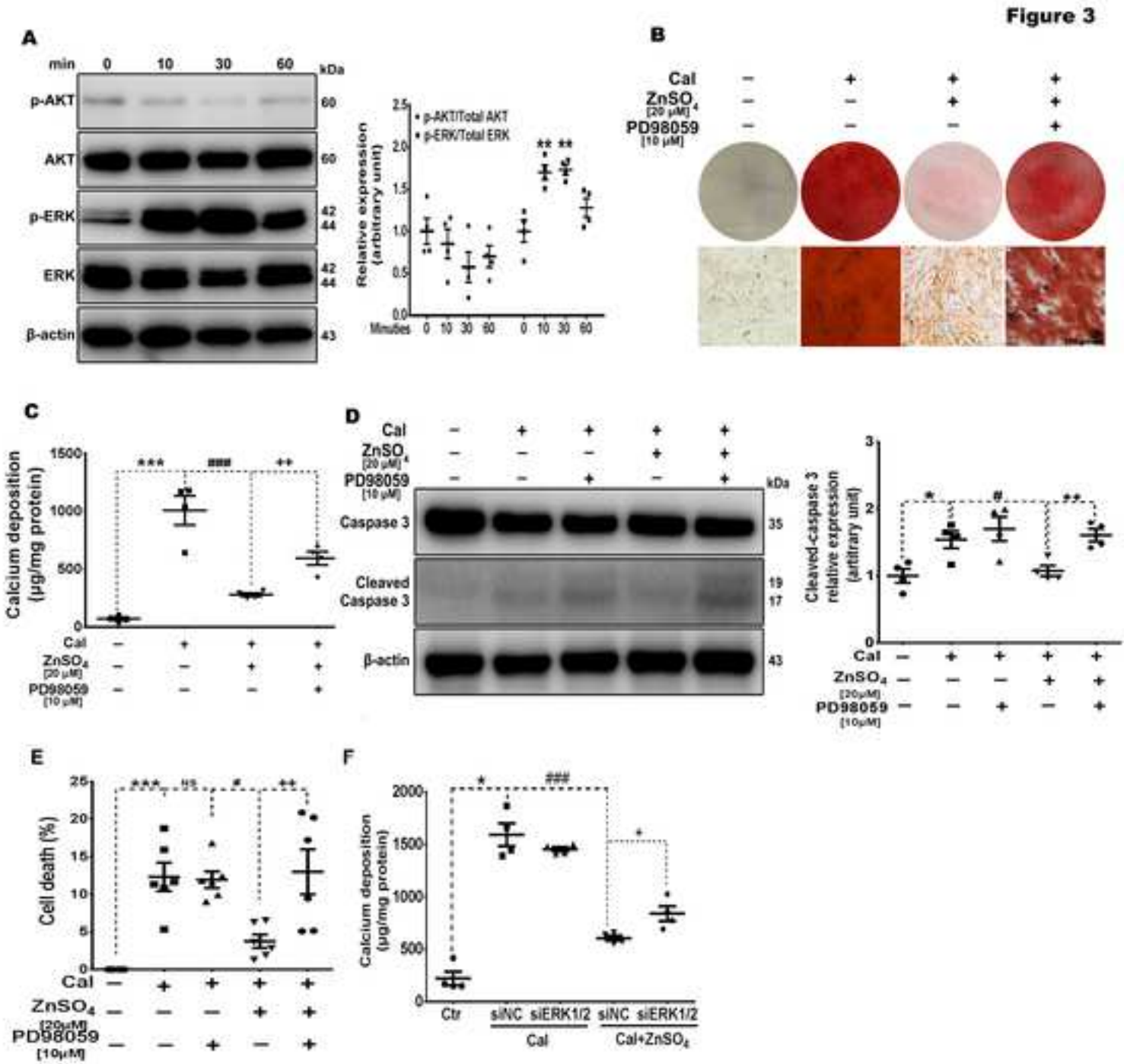
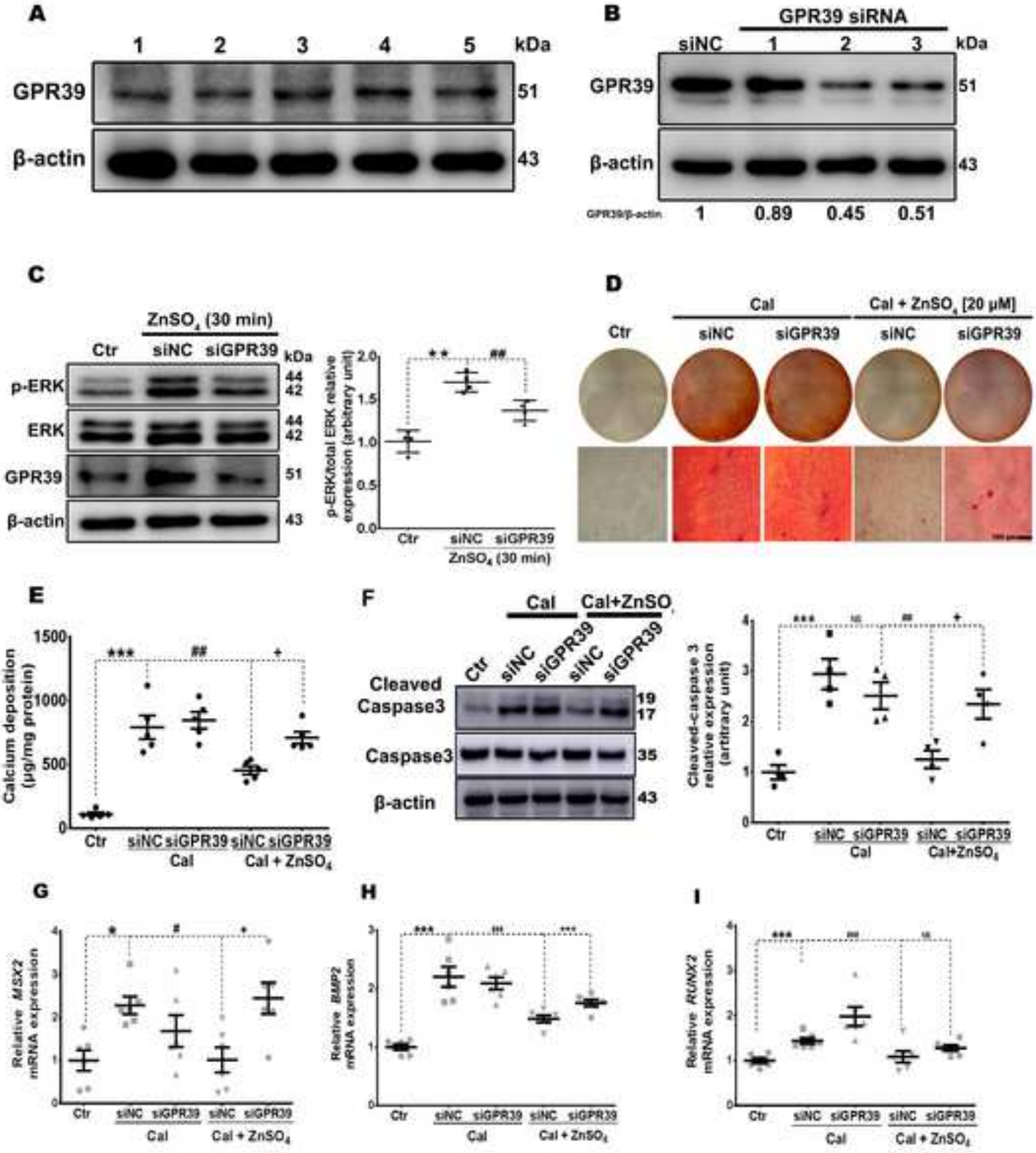


Figure 4



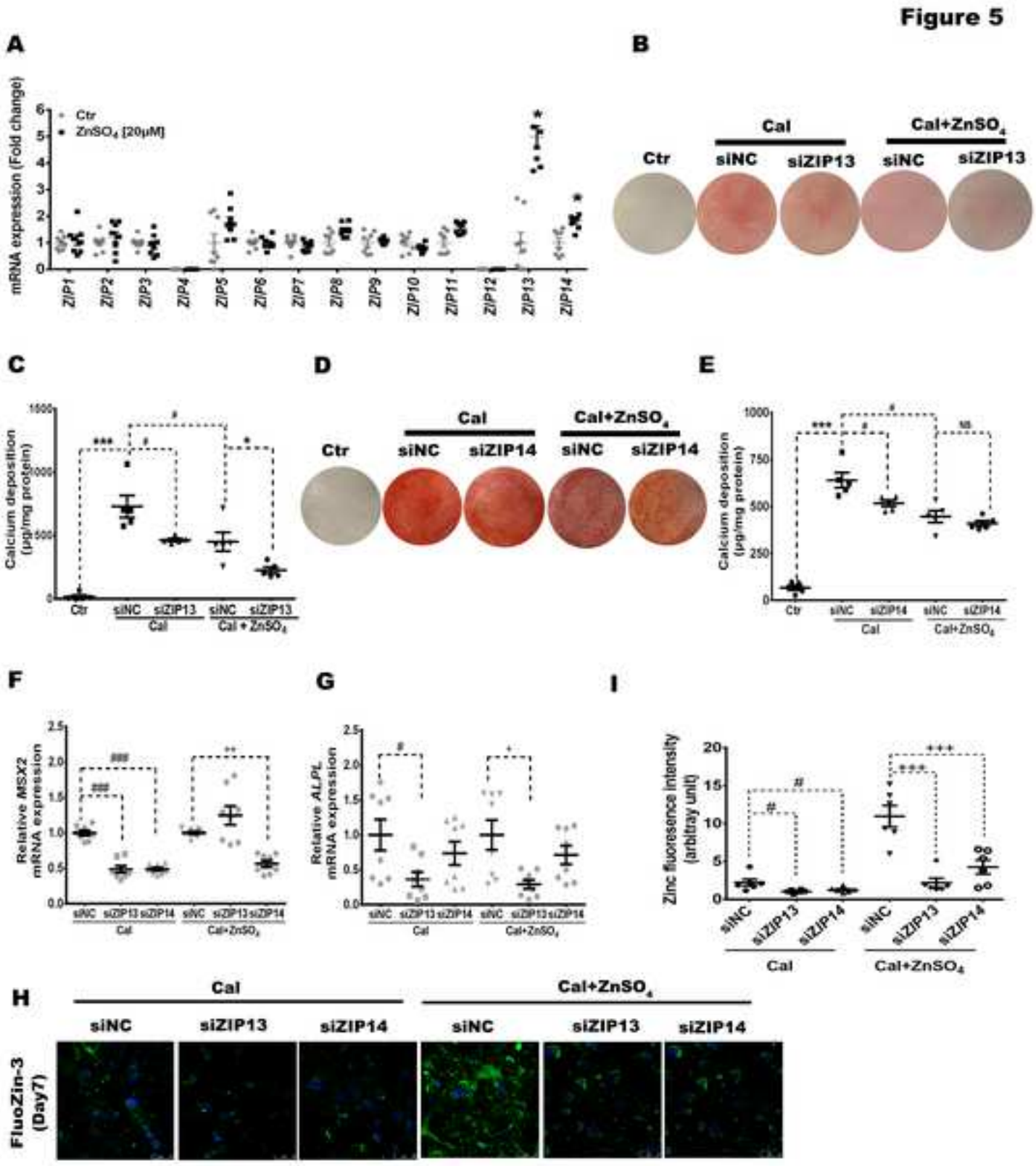


Figure 6

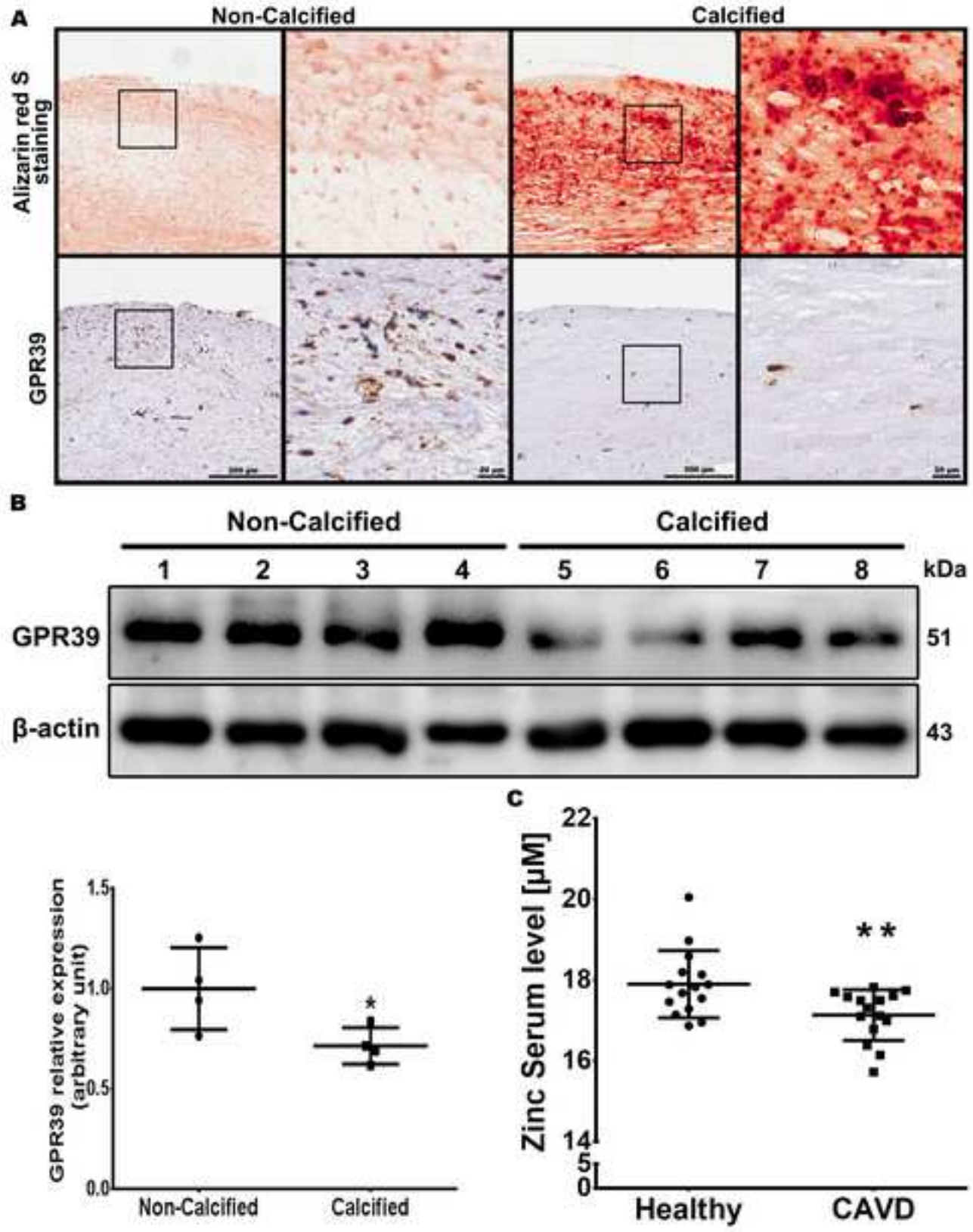
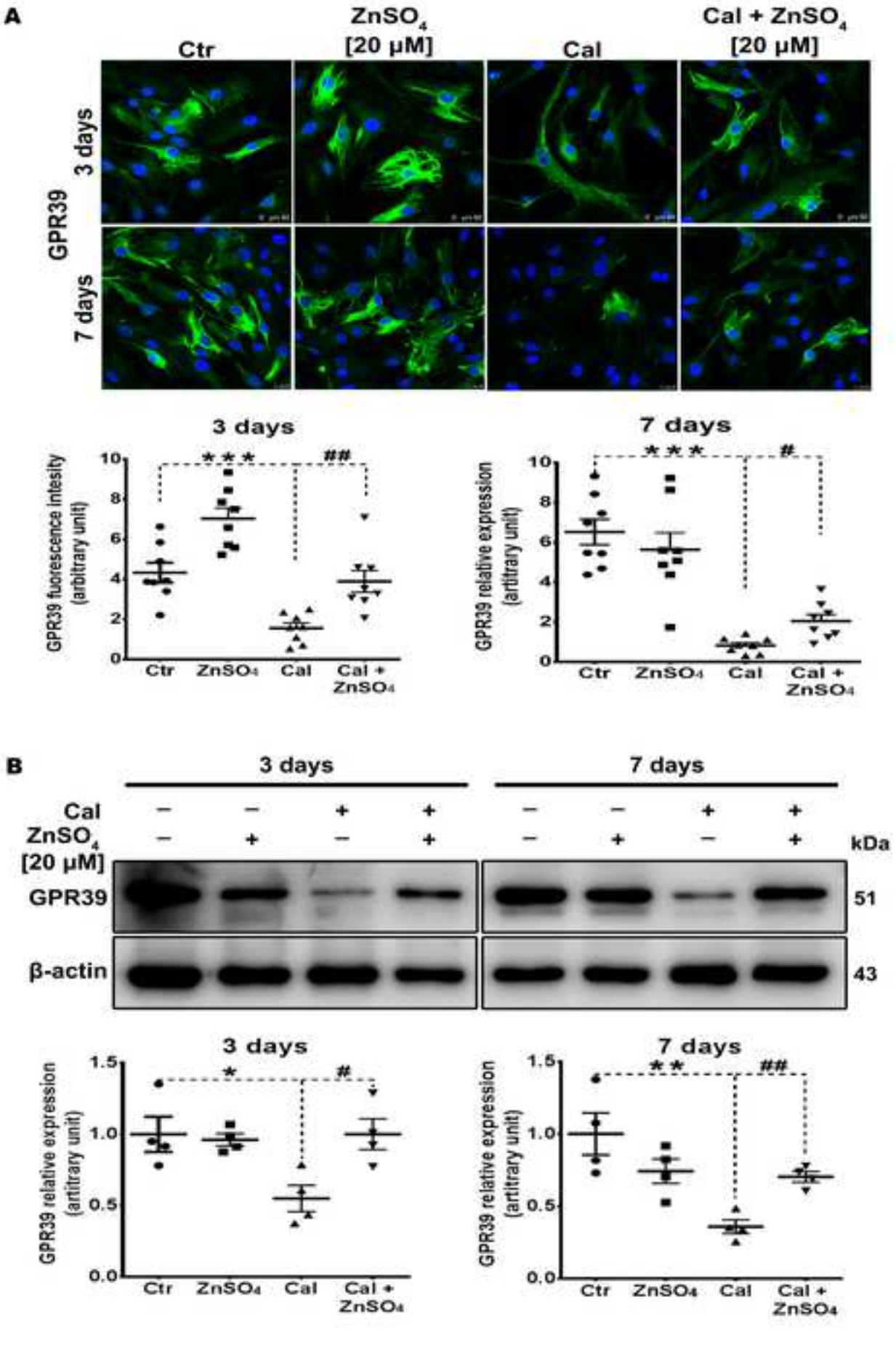
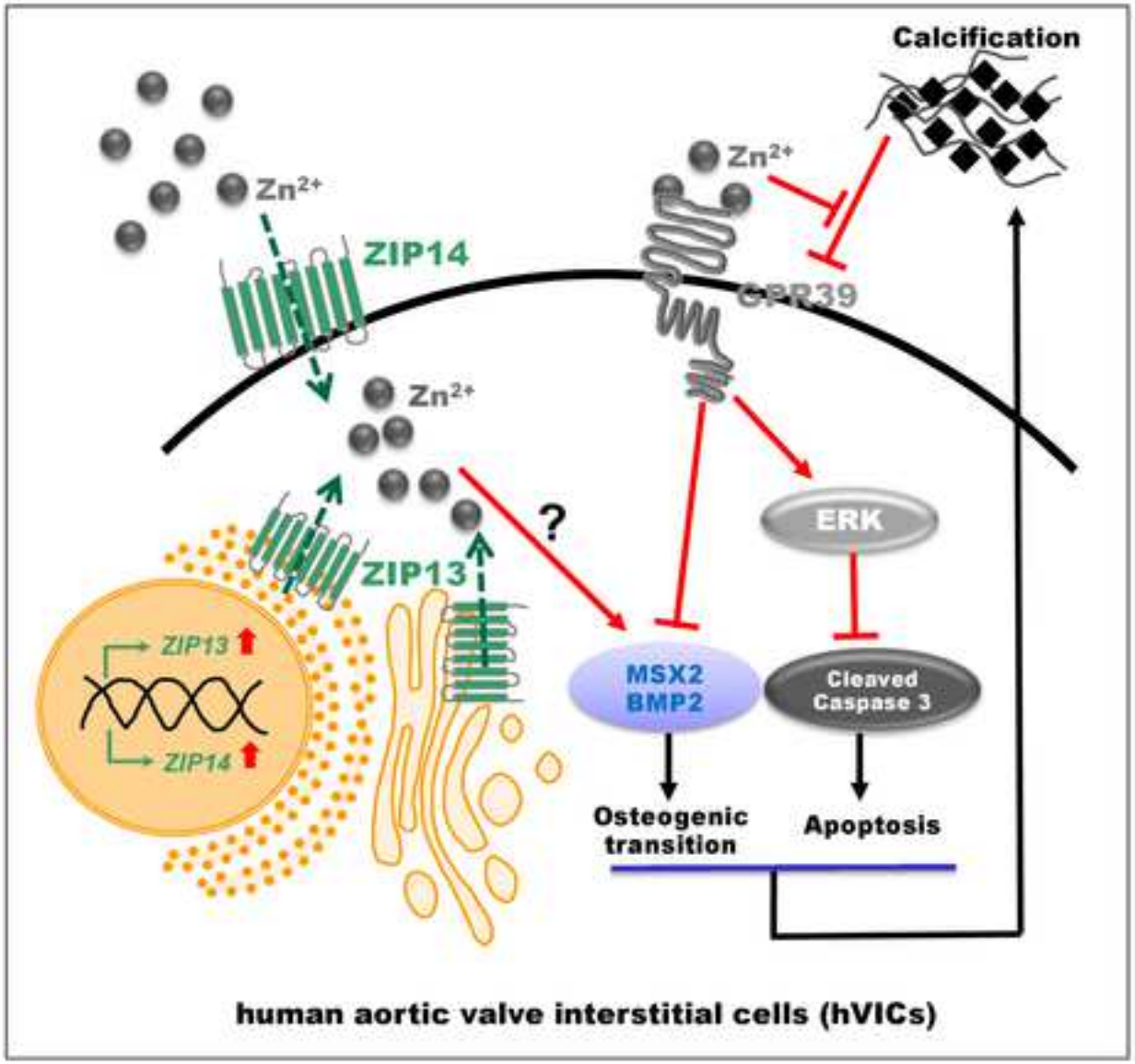


Figure 7



Graphical Abstract

**Graphic abstract**



# Zinc ameliorates human aortic valve calcification through GPR39 mediated ERK1/2 signaling pathway

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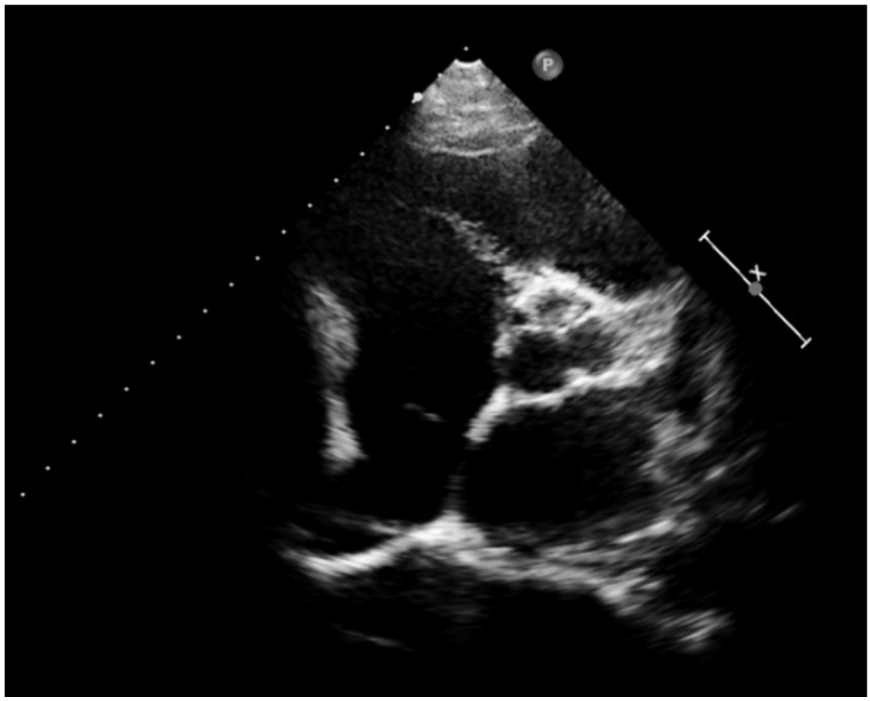
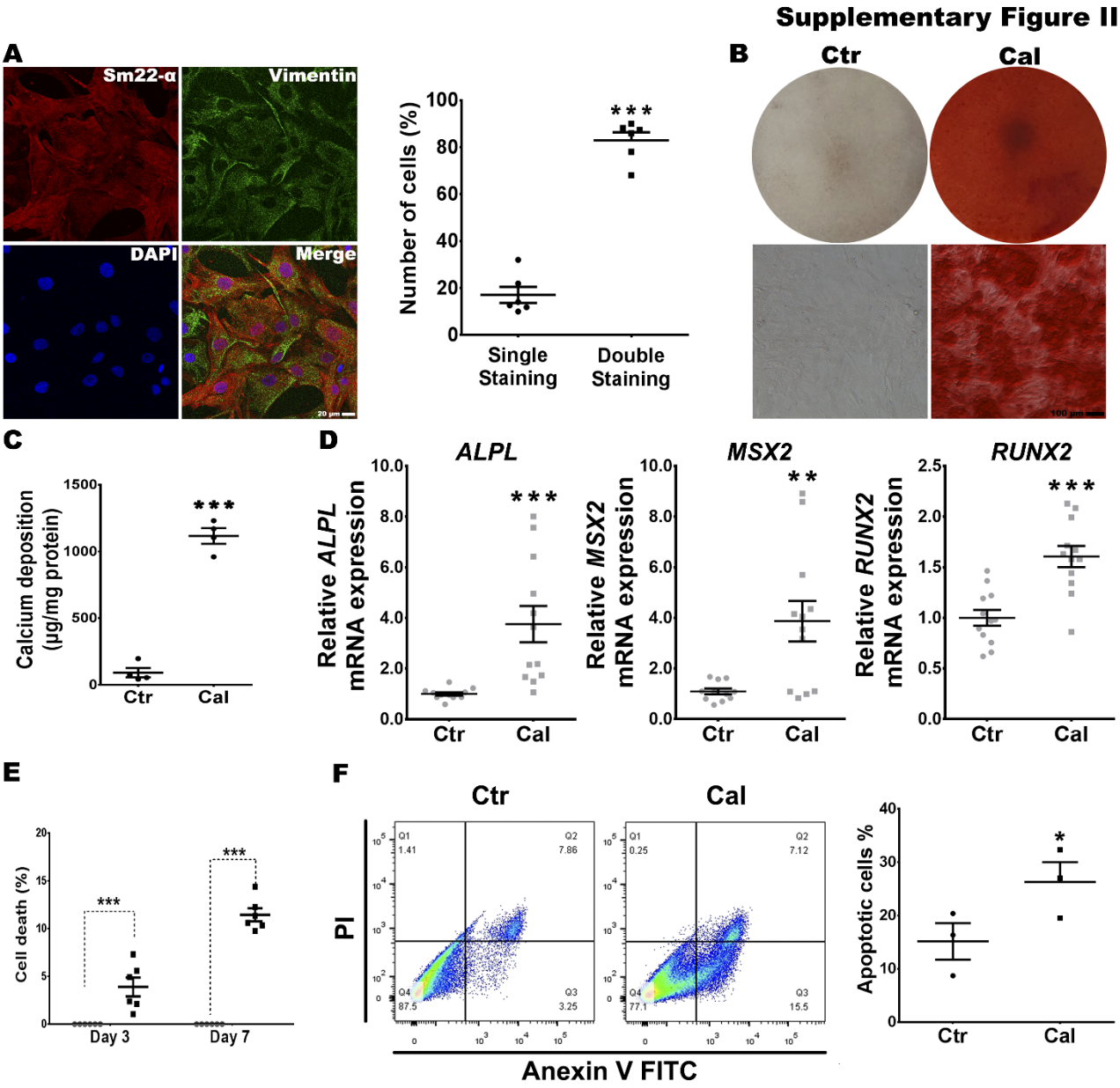
**Supplementary Figure I**

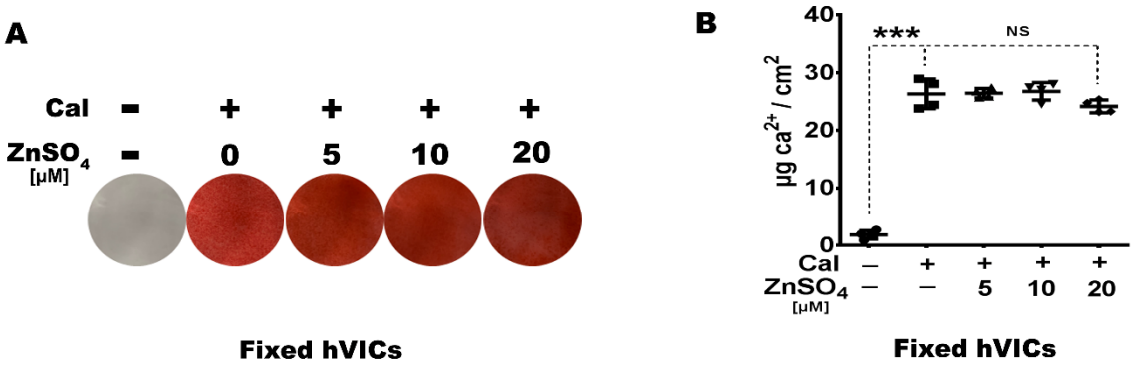


**Supplementary Figure I. A representative still image of echocardiograms of a patient with CAVD.**



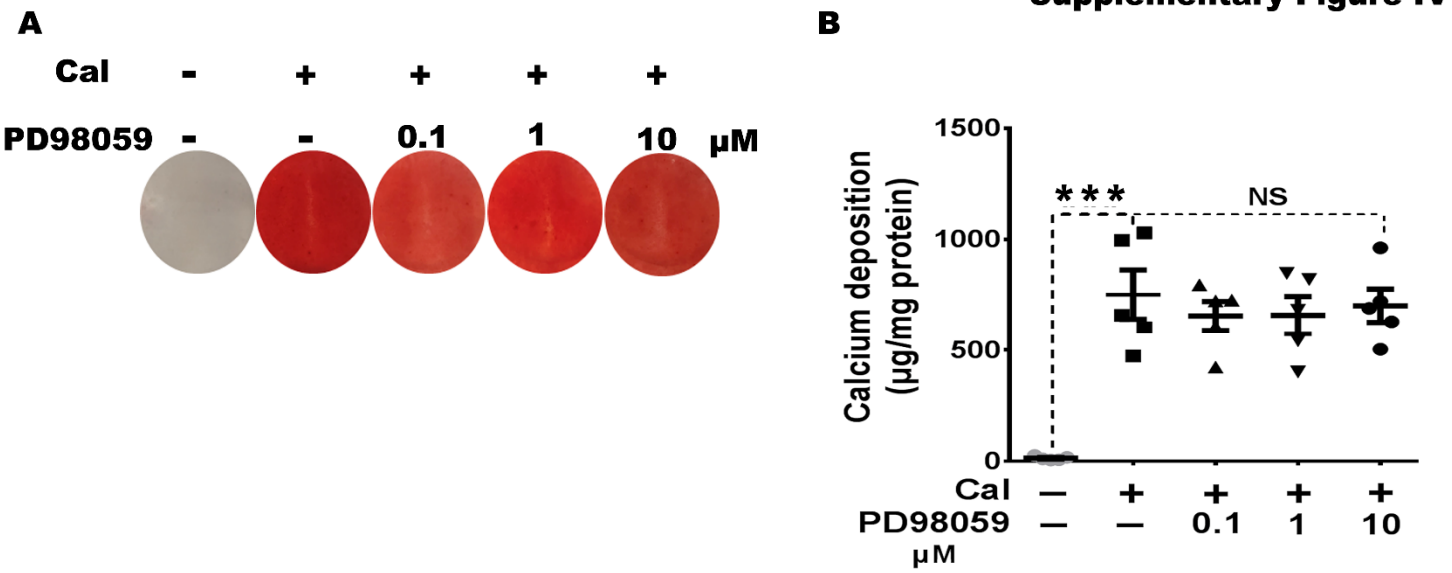

**Supplementary Figure II. Validation of hVICs *in vitro* calcification model**. hVICs were exposed to control (Ctr) or calcification media (Cal) for up to 7 days. **A.** Representative confocal images of vimentin and alpha 22-smooth muscle actin (Sm22-α) immunofluorescence staining (left panel) and quantitative analysis of percentage of cells expressing double valve interstitial cell markers (vimentin and Sm22-α) or single marker (right panel) (n=6). Scale bar 20 µm. **B**. Representative alizarin red S staining images at day 7 (n=3). Plate view (upper) and microscopic view (lower), scale bar 100 µm. **C.** Quantitative calcium assay at day 7 (n=4). **D.** Relative mRNA expression of ALPL, MSX2, and RUNX2 at day 2 (n=12), unpaired Student's t-test or Mann-Whitney test. **E.** Cell death at day 3 and day 7 (n=6). **F.** Flow cytometry analysis of Annexin V and Propidium Iodide (PI) staining (left panels) and quantification of the percentage of early apoptotic cells (Q3, right panel) at day 3 (n=3), unpaired Student's t-test. Results are presented as mean ± SEM.*p < 0.05, **p < 0.01, ***p < 0.001 compared to control.

**Supplementary Figure III**



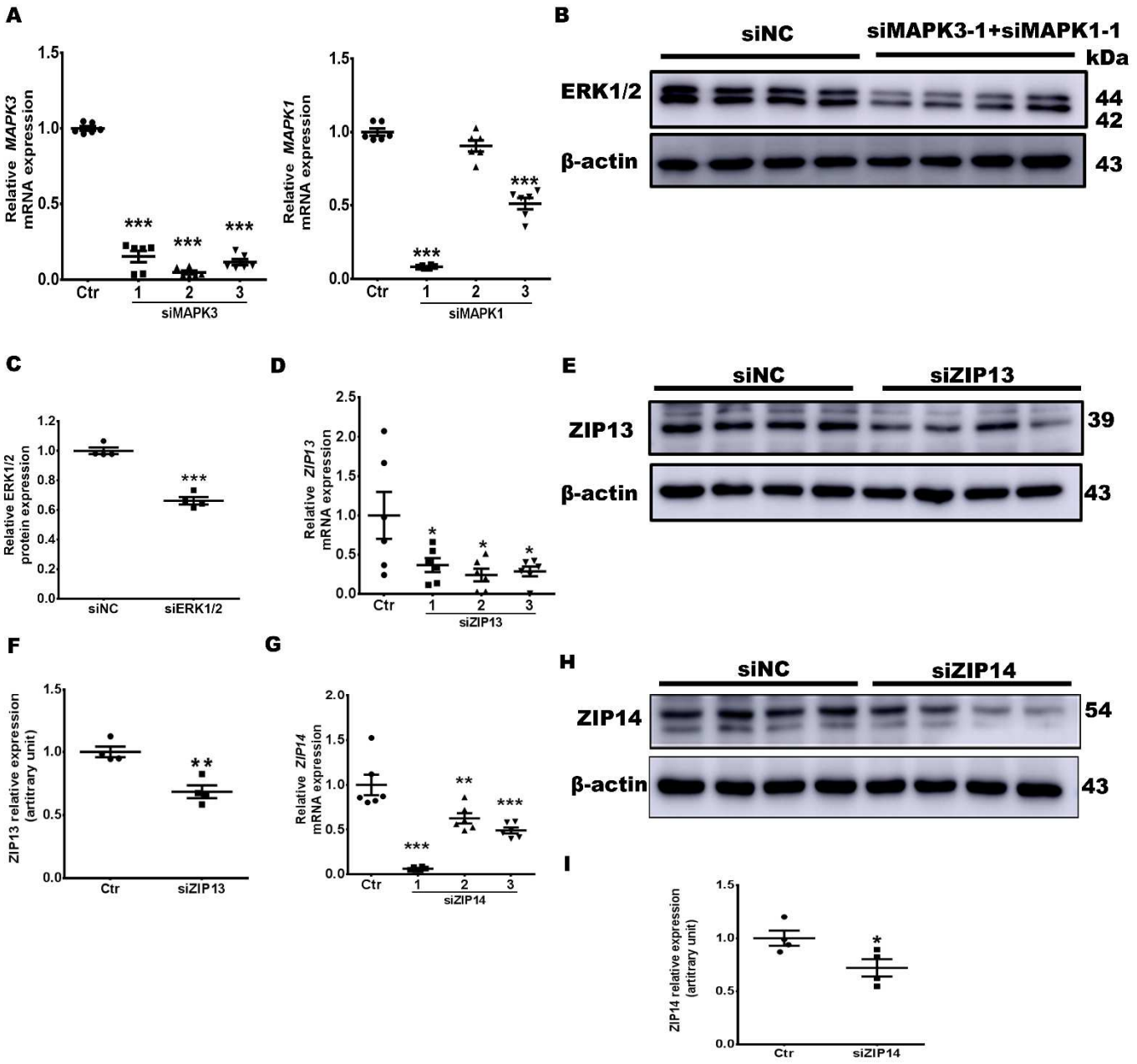

**Supplementary Figure III. Zinc does not alter calcium deposition on fixed hVICs.** hVICs were fixed with 4% PFA and then exposed to control (Ctr) or calcification media (Cal) without or with 20 µM of ZnSO4 treatment for 7 days. **A.** alizarin red staining. **B,** calcium quantitative assay (n=4). Results are presented as mean ± SEM. ANOVA by Bonferroni's test. ***p < 0.001 compared to control.

**Supplementary Figure IV**

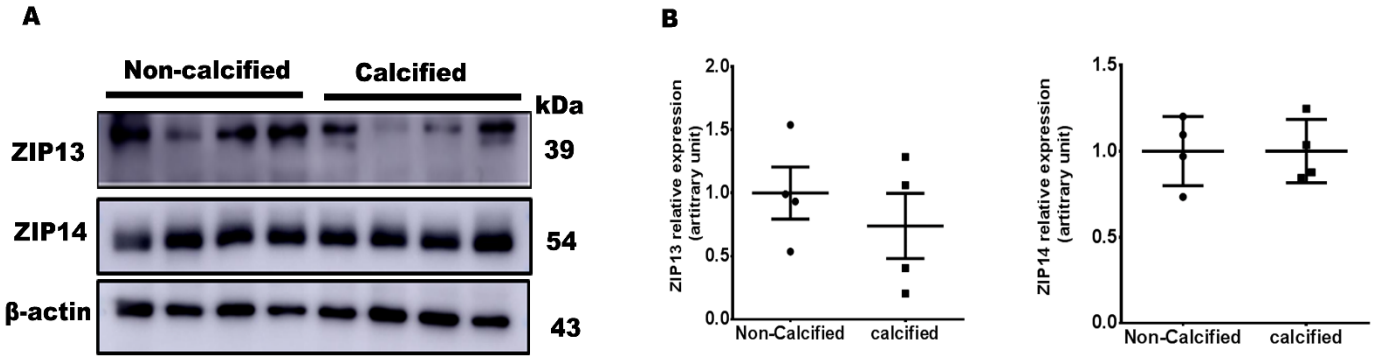



**Supplementary Figure IV. ERK1/2 inhibitor PD98058 did not alter hVIC *in vitro* calcification.** hVICs were exposed to control (Ctr) or calcification media (Cal) without or with 10 μM PD98059 for up to 7 days. **A.** Representative alizarin red S staining images at day 7 (n=3). **B.** Quantitative calcium assay at Day 7 (n=5).Results are presented as mean ± SEM. ANOVA by Bonferroni's test. ***p < 0.001 compared to control.

**Supplementary Figure V**




**Supplementary Figure V. Knockdown efficiency of siERK1/2, siZIP13 and siZIP14.** hVICs were transfected with siNC, siER1/2, siZIP13 or siZIP14 as indicated. **A.** Relative mRNA expression of ERK1 (*MAPK3) and* ERK2 *(MAPK1)* (n=6)*,* ANOVA by Bonferroni post-test. **B.** Western blotting images for ERK1/2 expression (n=4). **C.** Semi-quantitative analysis of expression of ERK1/2 (n=4), unpaired Student's t-test. **D.** Relative mRNA expression of ZIP13 (n=6)*,* ANOVA by Bonferroni post-test. **E.** Western blotting images for ZIP13 expression (n=4). **F.** Semi-quantitative analysis of expression of ZIP13 (n=4), unpaired Student's t-test. **G.** Relative mRNA expression of ZIP14 (n=6)*,* ANOVA by Bonferroni post-test. **H.** Western blotting images for ZIP14 expression (n=4). **I.** Semi-quantitative analysis of expression of ZIP14 (n=4), unpaired Student's t-test. Results are presented as mean ± SEM. ANOVA by Bonferroni's test. *p<0.05, **p<0.01, ***p < 0.001 compared to siNC.




**Supplementary Figure VI. ZIP13 and ZIP14 protein expression in human non-calcified and calcified aortic valves. A.** Western blotting images for ZIP13 and ZIP14 expression (n=4). **B.** Semi-quantitative analysis of expression of ZIP13 and ZIP14 (n=4), unpaired Student's t-test. Results are presented as mean ± SEM.

**Supplementary Table I. Clinical Characteristics of CAVD Patients for the isolation of hVICs.**

| Characteristics | CAVD (n=34) |
|---|---|
| **Age, years** | 52.15±2.37 |
| **Male, %** | 74 |
| **Smoking, %** | 24 |
| **Hypertension, %** | 38 |
| **Diabetes mellitus, %** | 3 |
| **Bicuspid aortic valves, %** | 21 |
| **BMI, kg/m$^2$** | 22.81±0.57 |
| **Triglycerides, mmol/L** | 1.49±0.18 |
| **LDL, mmol/L** | 2.94±0.18 |
| **HDL, mmol/L** | 1.24±0.16 |
| **Cholesterol, μmol/L** | 4.53±0.24 |
| **Statins,%** | 24 |
| **Beta-blocker, %** | 50 |
| **ACEI/ARB, %** | 26 |

CAVD indicates calcific aortic valve disease; BMI, body mass index; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; ACEI/ARB, Angiotensin Converting Enzyme Inhibitors/Angiotensin Receptor Blockers. Values are mean±SEM when appropriate.

**Supplementary Table II. Clinical Characteristics of Patients for the aortic valves studies.**

| Characteristics | Control (n=4) | CAVD (n=4) | P Value |
|---|---|---|---|
| **Age, years** | 56±3.58 | 65±3.09 | 0.098 |
| **Male, %** | 100 | 100 | 1 |
| **Smoking, %** | 0 | 25 | 1 |
| **Hypertension, %** | 100 | 0 | 0.0286 |
| **Diabetes mellitus, %** | 0 | 0 | 1 |
| **Bicuspid aortic valves, %** | 0 | 0 | 1 |
| **BMI, kg/m2** | 23.19±1.2 | 24.63±1.88 | 0.5416 |
| **Triglycerides, mmol/L** | 1.27±0.32 | 1.05±0.08 | 0.5011 |
| **LDL, mmol/L** | 2.84±0.30 | 2.88±0.43 | 0.9495 |
| **HDL, mmol/L** | 0.84±0.13 | 1.23±0.18 | 0.1527 |
| **Cholesterol, µmol/L** | 4.40±0.34 | 4.66±0.54 | 0.693 |
| **Statins, %** | 25 | 0 | 1 |
| **Beta-blocker, %** | 75 | 50 | 1 |
| **ACEI/ARB, %** | 75 | 0 | 0.1429 |

CAVD indicates calcific aortic valve disease; BMI, body mass index; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; ACEI/ARB, Angiotensin Converting Enzyme Inhibitors/ Angiotensin Receptor Blockers. Values are mean±SEM when appropriate. P values determined by the Student t test or Fisher exact test.

**Supplementary Table III. Clinical Characteristics of Patients for the Zinc Serum Level.**

| Characteristics | Control (n=15) | CAVD (n=15) | P Value |
|---|---|---|---|
| **Age, years** | 69.47±1.34 | 73.4±1.64 | 0.0737 |
| **Male, %** | 53 | 100 | 0.2451 |
| **Smoking, %** | NA | 86 | NA |
| **Hypertension, %** | NA | 60 | NA |
| **Diabetes mellitus, %** | NA | 27 | NA |
| **Bicuspid aortic valves, %** | NA | 7 | NA |
| **BMI, kg/m$^2$** | 23.97±0.5 | 21.69±0.7 | 0.0131 |
| **Triglycerides, mmol/L** | NA | 1.33±0.16 | NA |
| **LDL, mmol/L** | NA | 2.92±0.20 | NA |
| **HDL, mmol/L** | NA | 1.26±0.34 | NA |
| **Cholesterol, µmol/L** | NA | 4.58±0.23 | NA |
| **Statins, %** | NA | 73 | NA |
| **Beta-blocker, %** | NA | 67 | NA |
| **Marked increase in echogenicity of aortic valves** | 0 | 15 | <0.0001 |
| **LVEF, %** | 57.73±3.37 | 54.95±3.81 | 0.5903 |
| **AV, m/s** | 1.173±0.07 | 1.486±0.11 | 0.0186 |
| **AOD, cm** | 2.94±0.07 | 2.97±0.09 | 0.8076 |

CAVD indicates calcific aortic valve disease; BMI, body mass index; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; ACEI/ARB, Angiotensin Converting Enzyme Inhibitors/ Angiotensin Receptor Blockers; LVEF, left ventricular ejection fraction; AV, Aortic valve orifice velocity; AOD, Aortic diameter; Values are mean±SEM when appropriate. P values determined by the Student t test or Fisher exact test.

## Supplementary Table IV

STROBE Statement—Checklist of items that should be included in reports of ***case-control studies***

| | Item No | Recommendation | Page No |
|---|---|---|---|
| **Title and abstract** | 1 | (*a*) Indicate the study's design with a commonly used term in the title or the abstract | 1 |
| | | (*b*) Provide in the abstract an informative and balanced summary of what was done and what was found | 1 |
| **Introduction** | | | |
| Background/rationale | 2 | Explain the scientific background and rationale for the investigation being reported | 2-3 |
| Objectives | 3 | State specific objectives, including any prespecified hypotheses | 2-3 |
| **Methods** | | | |
| Study design | 4 | Present key elements of study design early in the paper | 3 |
| Setting | 5 | Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection | 3 |
| Participants | 6 | (*a*) Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls | 3 |
| | | (*b*) For matched studies, give matching criteria and the number of controls per case | N/A |
| Variables | 7 | Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable | 3, Supplementary table III |
| Data sources/ measurement | 8* | For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group | 3 |
| Bias | 9 | Describe any efforts to address potential sources of bias | 3 |
| Study size | 10 | Explain how the study size was arrived at | 3,10 |
| Quantitative variables | 11 | Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why | 3,10, Supplementary Methods |
| Statistical methods | 12 | (*a*) Describe all statistical methods, including those used to control for confounding | 6-7,10, Supplementary Methods |
| | | (*b*) Describe any methods used to examine subgroups and interactions | N/A |
| | | (*c*) Explain how missing data were addressed | 3,10, Supplementary Methods |
| | | (*d*) If applicable, explain how matching of cases and controls was addressed | 3,10 |
| | | (*e*) Describe any sensitivity analyses | N/A |
| **Results** | | | |
| Participants | 13* | (a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed | 3 |
| | | (b) Give reasons for non-participation at each stage | 3 |
| | | (c) Consider use of a flow diagram | N/A |

| | | | | |
|---|---|---|---|---|
| Descriptive data | | 14* | (a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders | Supplementary table III |
| | | | (b) Indicate number of participants with missing data for each variable of interest | 3, Supplementary table III |
| Outcome data | | 15* | Report numbers in each exposure category, or summary measures of exposure | Supplementary table III |
| Main results | | 16 | (*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included | 10, Supplementary table III, Figure 6C |
| | | | (*b*) Report category boundaries when continuous variables were categorized | 10, Supplementary table III, Figure 6C |
| | | | (*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period | N/A |
| Other analyses | 17 | | Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses | N/A |
| **Discussion** | | | | |
| Key results | 18 | | Summarise key results with reference to study objectives | 10-14 |
| Limitations | 19 | | Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias | 13-14 |
| Interpretation | 20 | | Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence | 10-14 |
| Generalisability | 21 | | Discuss the generalisability (external validity) of the study results | 10-14 |
| **Other information** | | | | |
| Funding | 22 | | Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based | 14 |

*Give information separately for cases and controls.

**Note:** An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.

**Supplementary Table V. The qPCR primer sequences for target genes**

| Gene | Forward primer (5'-3') | Reverse primer (5'-3') |
|---|---|---|
| **RUNX2** | GCCTTCCACTCTCAGTAAGAAGA | GCCTGGGGTCTGAAAAAGGG |
| **ALPL** | ACTGGTACTCAGACAACGAGAT | ACGTCAATGTCCCTGATGTTATG |
| **MSX2** | TGGATGCAGGAACCCGG | AGGGCTCATATGTCTTGGCG |
| **BMP2** | TTCGGCCTGAAACAGAGACC | CCTGAGTGCCTGCGATACAG |
| **GAPDH** | GAGTCAACGGATTTGGTCGT | GACAAGCTTCCCGTTCTCAG |
| **ZIP1** | ACTACCTGGCTGCCATAGATG | GCCCTGACTGCTCCTTGTAAG |
| **ZIP2** | TCACAGATTCAGAAGTTCATGGT | GCTCTCCATAGGGATACTCCA |
| **ZIP3** | CTCGGCCACATCAGCAC | TTGAAGGTCTCCAGGTCGAT |
| **ZIP4** | AAGATGGCCTGCGTAGATA | TGCTGCTGGAACACAAAG |
| **ZIP5** | GGGGCTGTCAGTGCTCGGAG | TCCGGATCCAAGTTGCGTGTT |
| **ZIP6** | CAACTATCTCTGTCCAGCCATC | CCACCAACCCAGGCTATTT |
| **ZIP7** | CTCTACTTCAGATCTTGCTCAGTT | TGGTGAGAATGAGGTTCAAGAG |
| **ZIP8** | TGCTACCCAAATAACCAGCTCC | ACAGGAATCCATATCCCCAAACT |
| **ZIP9** | TCTCTGGCTATGTTGGTGGGA | CCAGCACCCAAAACAGTCAC |
| **ZIP10** | ACACCAGATTCTGACTGGCTT | TAGGAGGGGATTCTTGTTGGC |
| **ZIP11** | TGCTGGGGACCTTCTTCAC | GCCAAGACTTCCATCTAAGATCC |
| **ZIP12** | TTTCCTGGGATCAGACCTGCT | GTTGGTCCTTGGGTAAGTGGC |
| **ZIP13** | TCCTGGGTTCCCTCATGGT | AGATGCAGAAACACATTGCCC |
| **ZIP14** | GGCTGCTGCTCTACTTCATAG | TCCAGAGGGTTGAAACCAAAT |
| **MAPK1** | TACACCAACCTCTCGTACATCG | CATGTCTGAAGCGCAGTAAGATT |
| **MAPK3** | CTACACGCAGTTGCAGTACAT | CAGCAGGATCTGGATCTCCC |
| **TNFα** | GAGGCCAAGCCCTGGTATG | CGGGCCGATTGATCTCAGC |
| **TGFβ1** | CAATTCCTGGCGATACCTCAG | GCACAACTCCGGTGACATCAA |

**Supplementary Table VI. siRNA sequences for gene silencing**

| siRNA | Sequence (5'-3') |
|---|---|
| GPR39 siRNA 1 | AGUGACUGCUCCCAAAUCA |
| GPR39 siRNA 2 | GACAGACCACAUGGUGAGU |
| GPR39 siRNA 3 | AUGCCCAUGGAGUUCUACA |
| MAPK1 siRNA 1 | GAACATCATTGGAATCAAT |
| MAPK1 siRNA 2 | GCTACACCAACCTCTCGTA |
| MAPK1 siRNA 3 | GCACCAACCATCGAGCAAA |
| MAPK3 siRNA 1 | CCTCCAACCTGCTCATCAA |
| MAPK3 siRNA 2 | GACCGGATGTTAACCTTTA |
| MAPK3 siRNA 3 | GCTACACGCAGTTGCAGTA |
| ZIP13 siRNA 1 | GCTTCCTTGTGAGCAAGAA |
| ZIP13 siRNA 2 | CTGACCTCTTGGAAGAAGA |
| ZIP13 siRNA 3 | CTGGCCAACACCATCGATA |
| ZIP14 siRNA1 | GGAGGAATGTTCTTGTATA |
| ZIP14 siRNA2 | GGTGTCCGCTAACTCTGATA |
| ZIP14 siRNA3 | GGAGTTCCCACATGAGCTA |

**Supplementary Methods**

All statistical testing was 2-sided, with $P < 0.05$ considered significant. Analyses were performed using IBM SPSS Statistics 19.0 (IBM Corporation, Chicago, USA). The comparison of the baseline variables of interest were performed according to the patients with CAVD or not. Continuous variables, such as age, Zn and BMI, were denoted as mean ± standard deviation, and student's t test was conducted to compare the variables. Category variables, such as sex, were denoted as number (proportions) of female, and $\chi^2$ test was conducted to compare the variables. **Table 1**showed that there is a significant lower Zn concentration (17.1±0.6 vs 17.9 ± 0.8 μmol/L, P=0.014) and lower BMI (21.7 ± 2.7 vs 23.9 ± 1.9 kg/m$^2$, P=0.013) in CAVD group. There is no significant difference of sex and age between the two groups.

**Table 1 baseline characteristics of different groups**

| Variables | CAVD (n =15) | non-CAVD (n =15) | P value |
|---|---|---|---|
| Age, Mean (SD), y | 73.4 (6.4) | 69.5 (5.2) | 0.074 |
| Female, No. (%) | 3 (20) | 7 (47) | 0.13 |
| Zn, mean (SD), μ mol/L | 17.1 (0.6) | 17.9 (0.8) | 0.014 |
| BMI, mean (SD), kg/m$^2$ | 21.7(2.7) | 23.9 (1.9) | 0.013 |

**Table 2** showed the univariate and multivariate analyses of the variables predicting CAVD. The increasing Zn and BMI showed to be a significant protect factor of CAVD from the univariate analyses. After adjusting all the 4 variables of baseline, only the increasing BMI is the independent protective factor with a statistical significance (OR=0.598; C.I.: 0.371~0.966; P=0.035). There is an apparent trend in the final analyses to show the relationship between increasing Zn and CAVD

(OR=0.128; C.I.: 0.015~1.090; P=0.060). We believe that this negative result attribute to the small participates number.

**Table 2 univariable and multivariable analysis**

| | Univariable analysis | | | Multivariable analysis | | |
|---|---|---|---|---|---|---|
| | OR | 95% C.I. | P | OR | 95% C.I. | P |
| Age increase 1 | 1.132 | [0.983~1.303] | 0.084 | 1.111 | [0.896~1.378] | 0.339 |
| Male | 3.500 | [0.692~17.714] | 0.130 | 3.740 | [0.394~35.482] | 0.251 |
| Zn increase 1 | 0.148 | [0.026~0.826] | 0.029 | 0.128 | [0.015~1.090] | 0.060 |
| BMI increase 1 | 0.653 | [0.449~0.950] | 0.026 | 0.598 | [0.371~0.966] | 0.035 |

Furthermore, considering that there is no difference in the baseline and univariate analysis in age and sex and only 15 patients were diagnosed with CAVD, it is rational to include no more than 2 variables in the final multivariate analyses according to the statistical rule: the events per variable should be 10 or more. We performed a forward stepwise multivariate logistic analysis (likelihood ratio) to get a more convincible result. Only Zn and BMI were included in the final model (**Table 3**). From this result, increasing Zn was proved to be a significant protective factor of CAVD after adjusted the BMI (OR=0.135; C.I.: 0.022~0.815; P=0.029).

**Table 3 univariable and multivariable analysis of forward stepwise logistic analysis**

| | Univariable analysis | | | Multivariable analysis | | |
|---|---|---|---|---|---|---|
| | OR | 95% C.I. | P | OR | 95% C.I. | P |
| Age increase 1 | 1.132 | [0.983~1.303] | 0.084 | | | |
| Male | 3.500 | [0.692~17.714] | 0.130 | | | |
| Zn increase 1 | 0.148 | [0.026~0.826] | 0.029 | 0.135 | [0.022~0.815] | 0.029 |
| BMI increase 1 | 0.653 | [0.449~0.950] | 0.026 | 0.603 | [0.377~0.966] | 0.035 |

Figure 1

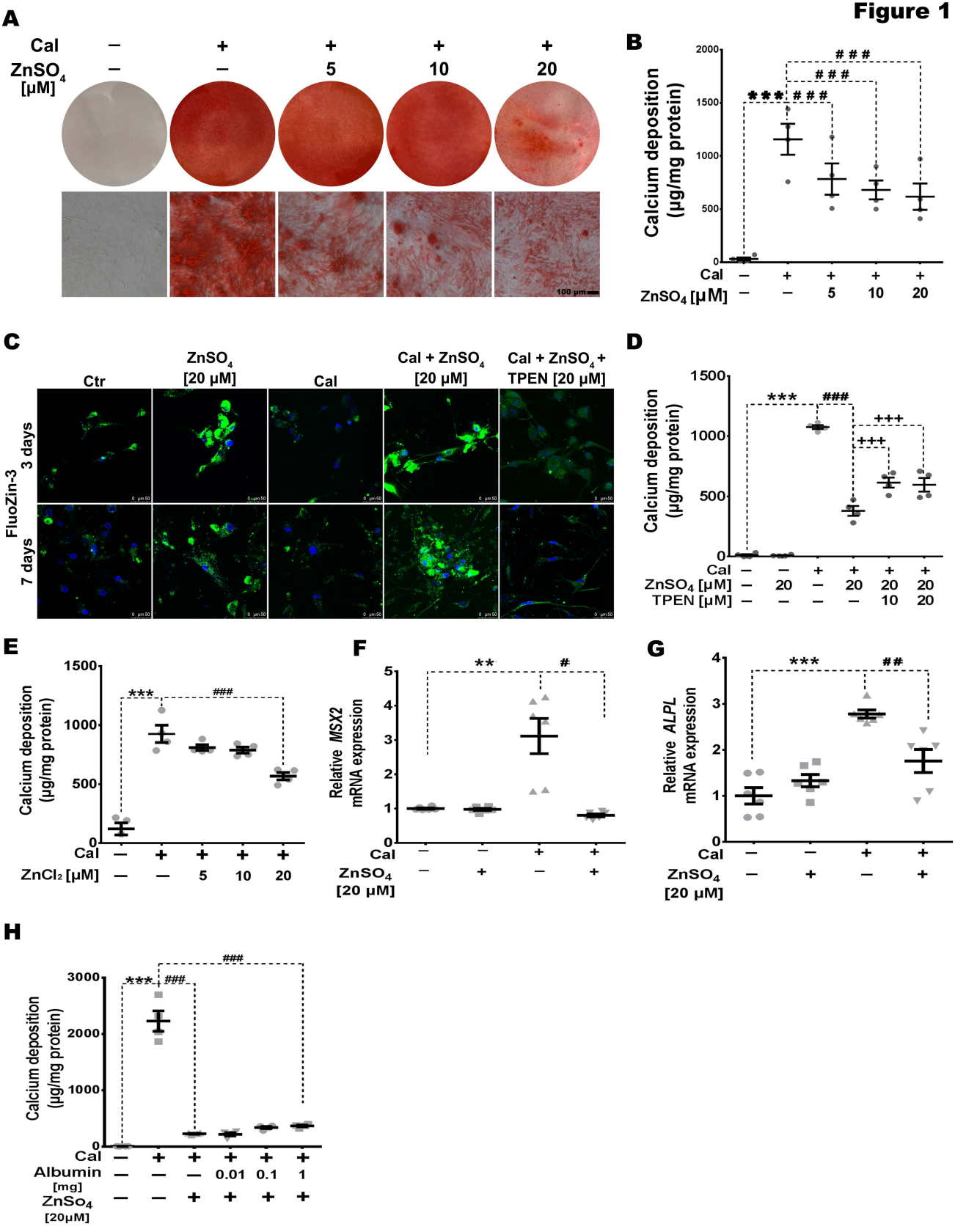

A
Cal
ZnSO4 [μM]
B
Calcium deposition (μg/mg protein)
C
Ctr
ZnSO4 [20 μM]
Cal
Cal + ZnSO4 [20 μM]
Cal + ZnSO4 + TPEN [20 μM]
FluoZin-3
3 days
7 days
D
TPEN [μM]
E
ZnCl2 [μM]
F
Relative MSX2 mRNA expression
G
Relative ALPL mRNA expression
H
Albumin [mg]

**Figure 2**

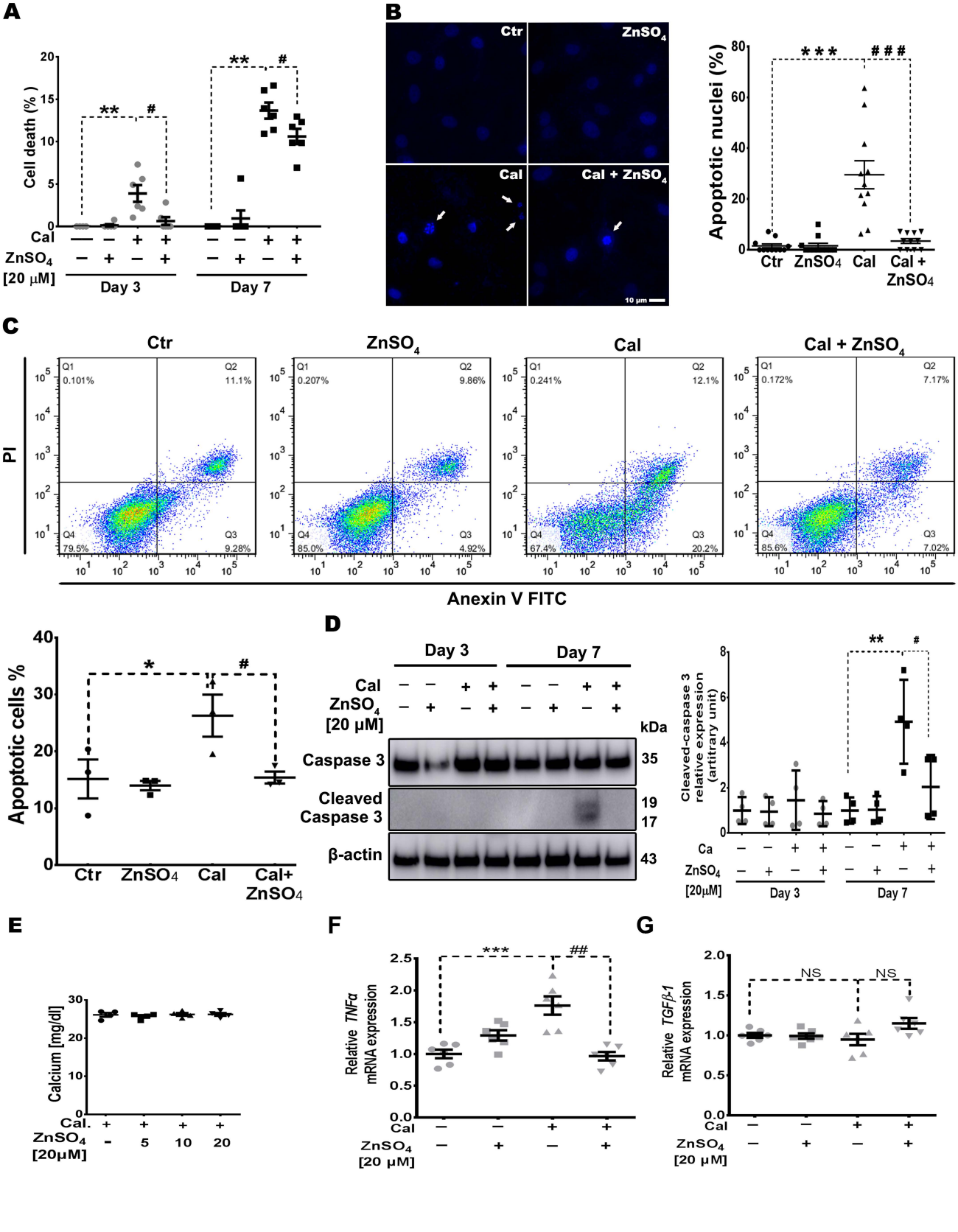
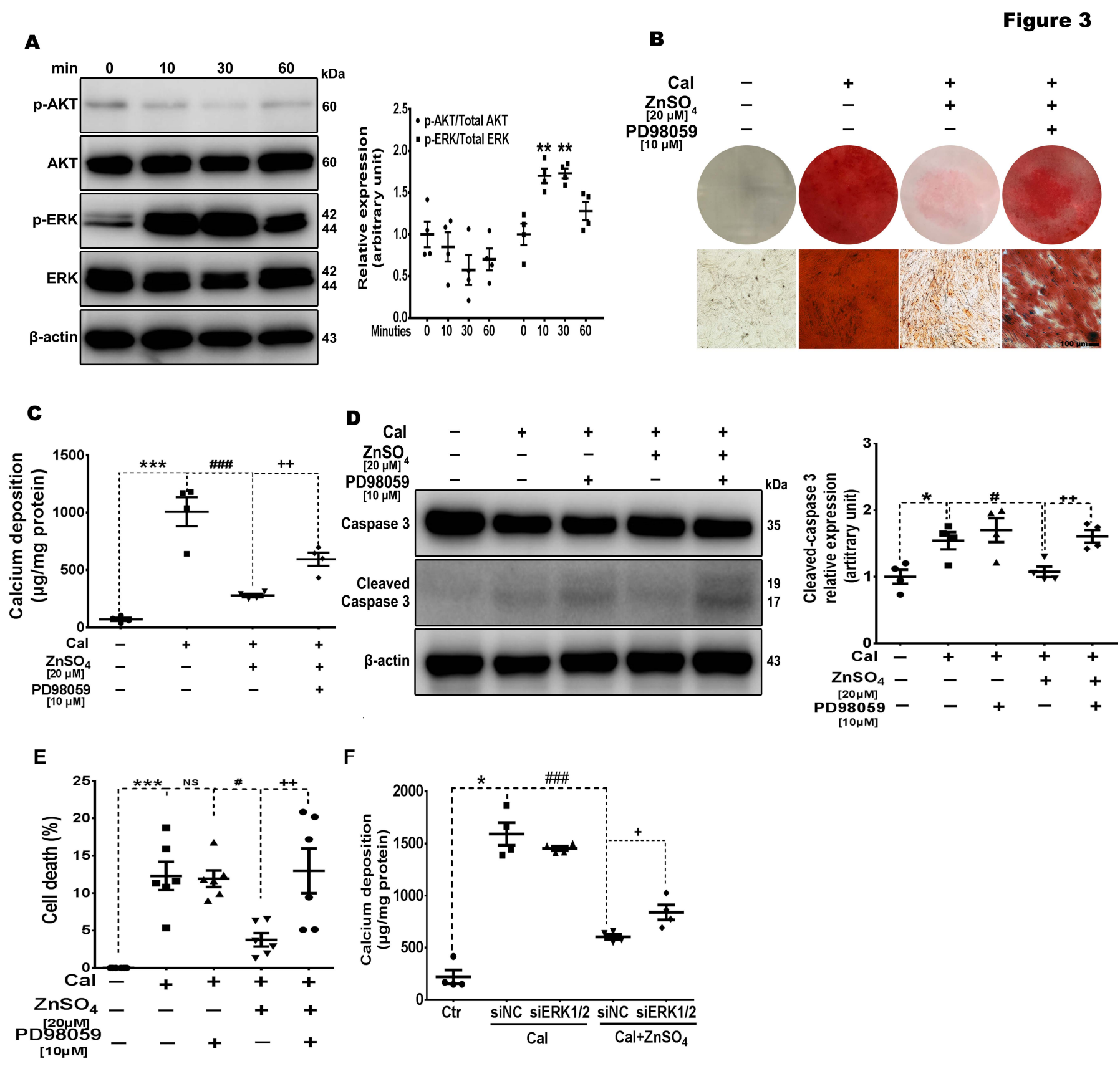

A
Cell death (% )
**
#
Cal
ZnSO4
[20 μM]
Day 3
Day 7
B
Ctr
ZnSO4
Cal
Cal + ZnSO4
10 μm
Apoptotic nuclei (%)
***
###
Ctr
ZnSO4
Cal
Cal + ZnSO4
C
Ctr
ZnSO4
Cal
Cal + ZnSO4
PI
Anexin V FITC
Q1 0.101%
Q2 11.1%
Q4 79.5%
Q3 9.28%
Q1 0.207%
Q2 9.86%
Q4 85.0%
Q3 4.92%
Q1 0.241%
Q2 12.1%
Q4 67.4%
Q3 20.2%
Q1 0.172%
Q2 7.17%
Q4 85.6%
Q3 7.02%
Apoptotic cells %
*
#
Ctr
ZnSO4
Cal
Cal+ ZnSO4
D
Day 3
Day 7
Cal
ZnSO4
[20 μM]
kDa
Caspase 3
35
Cleaved Caspase 3
19
17
β-actin
43
Cleaved-caspase 3 relative expression (artitrary unit)
**
#
Ca
ZnSO4
[20μM]
Day 3
Day 7
E
Calcium [mg/dl]
Cal.
ZnSO4
[20μM]
5
10
20
F
***
##
Relative TNFα mRNA expression
Cal
ZnSO4
[20 μM]
G
NS
NS
Relative TGFβ-1 mRNA expression
Cal
ZnSO4
[20 μM]

Figure 3


A
min 0 10 30 60 kDa
p-AKT 60
AKT 60
p-ERK 42 44
ERK 42 44
β-actin 43
p-AKT/Total AKT
p-ERK/Total ERK
Relative expression (arbitrary unit)
Minuties 0 10 30 60 0 10 30 60
B
Cal
ZnSO4 [20 μM]
PD98059 [10 μM]
100 μm
C
Calcium deposition (μg/mg protein)
***
###
++
Cal
ZnSO4 [20 μM]
PD98059 [10 μM]
D
Caspase 3 35
Cleaved Caspase 3 19 17
β-actin 43
Cleaved-caspase 3 relative expression (artitrary unit)
*
#
++
E
Cell death (%)
***
NS
#
++
F
Calcium deposition (μg/mg protein)
*
###
+
Ctr
siNC siERK1/2 siNC siERK1/2
Cal
Cal+ZnSO4

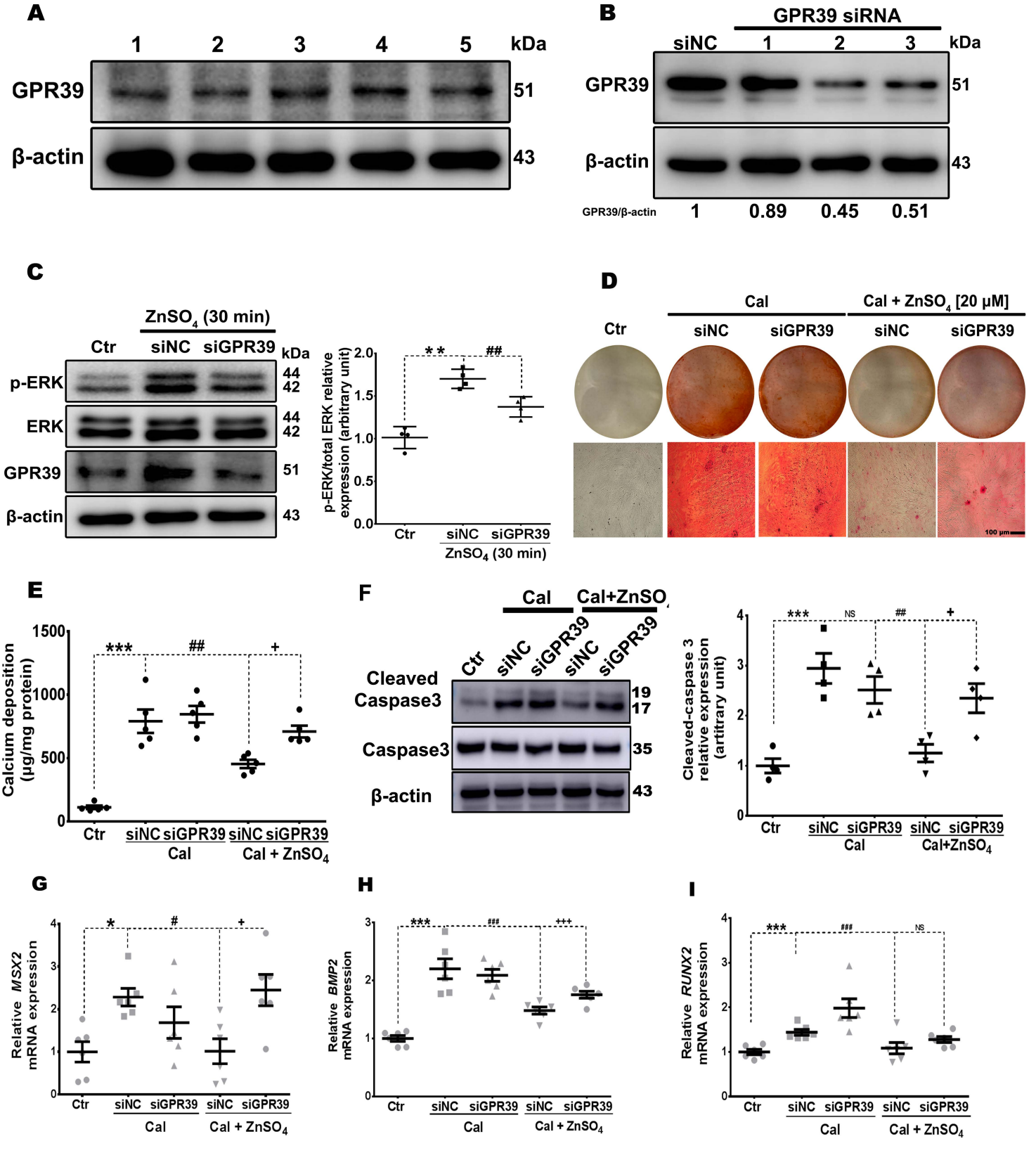

A
1
2
3
4
5
kDa
GPR39
51
β-actin
43
B
GPR39 siRNA
siNC
1
2
3
kDa
GPR39
51
β-actin
43
GPR39/β-actin
1
0.89
0.45
0.51
C
ZnSO4 (30 min)
Ctr
siNC
siGPR39
kDa
p-ERK
44
42
ERK
44
42
GPR39
51
β-actin
43
p-ERK/total ERK relative expression (arbitrary unit)
2.0
1.5
1.0
0.5
0.0
**
##
Ctr
siNC
siGPR39
ZnSO4 (30 min)
D
Cal
Cal + ZnSO4 [20 μM]
Ctr
siNC
siGPR39
siNC
siGPR39
100 μm
E
Calcium deposition (μg/mg protein)
1500
1000
500
0
***
##
+
Ctr
siNC
siGPR39
siNC
siGPR39
Cal
Cal + ZnSO4
F
Cal
Cal+ZnSO4
Ctr
siNC
siGPR39
siNC
siGPR39
Cleaved Caspase3
19
17
Caspase3
35
β-actin
43
Cleaved-caspase 3 relative expression (arbitrary unit)
4
3
2
1
0
***
NS
##
+
Ctr
siNC
siGPR39
siNC
siGPR39
Cal
Cal+ZnSO4
G
Relative MSX2 mRNA expression
4
3
2
1
0
*
#
+
Ctr
siNC
siGPR39
siNC
siGPR39
Cal
Cal + ZnSO4
H
Relative BMP2 mRNA expression
3
2
1
0
***
##
+++
Ctr
siNC
siGPR39
siNC
siGPR39
Cal
Cal + ZnSO4
I
Relative RUNX2 mRNA expression
4
3
2
1
0
***
##
NS
Ctr
siNC
siGPR39
siNC
siGPR39
Cal
Cal + ZnSO4

Figure 5

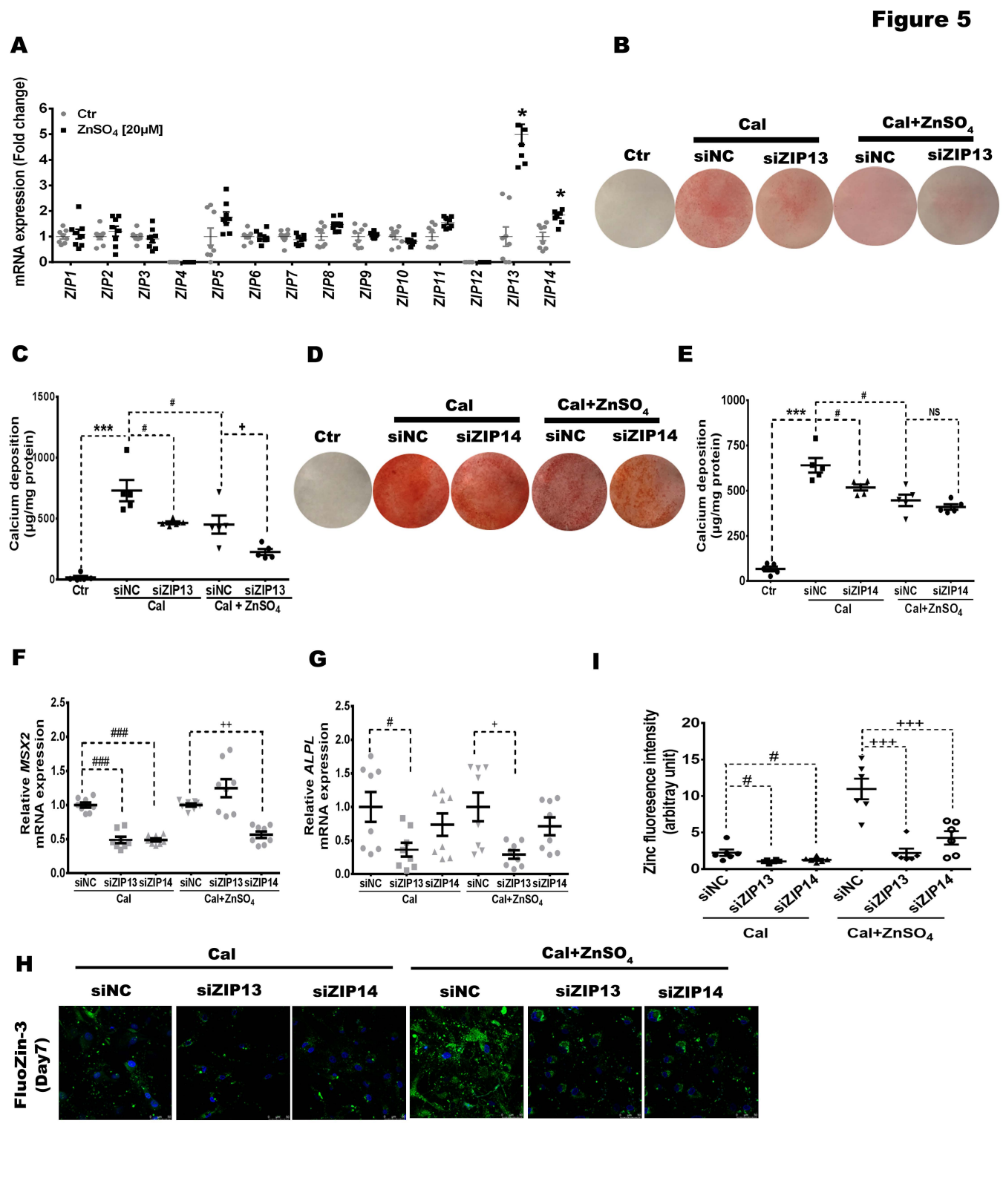
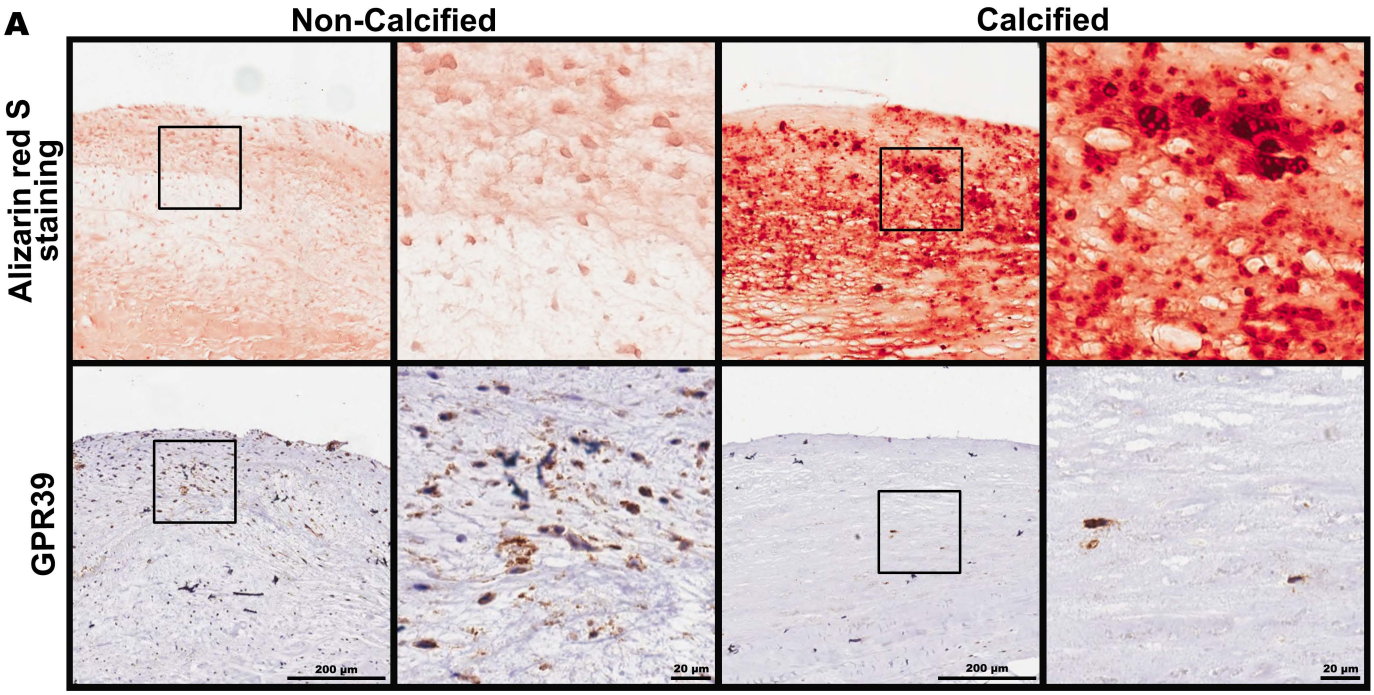
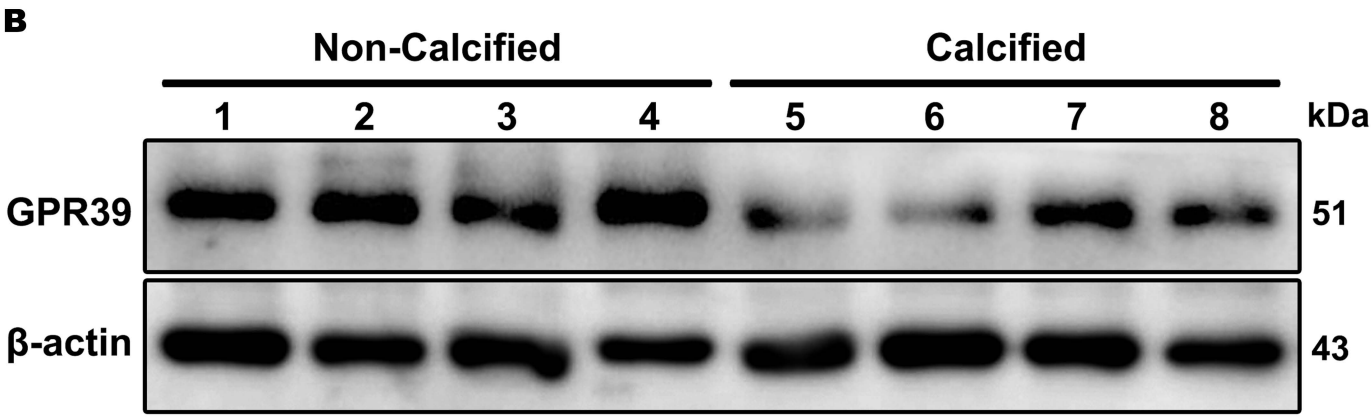
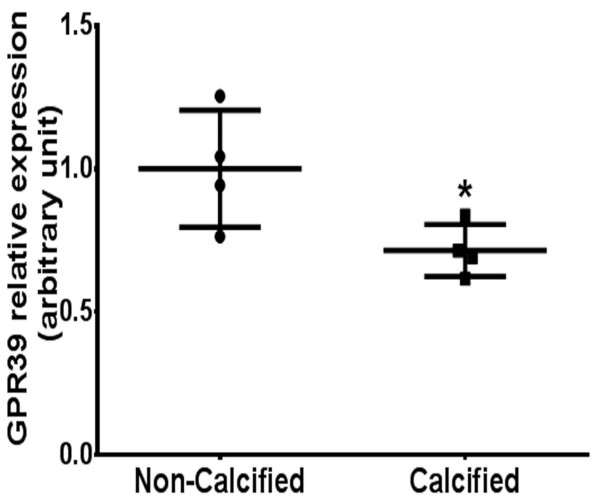
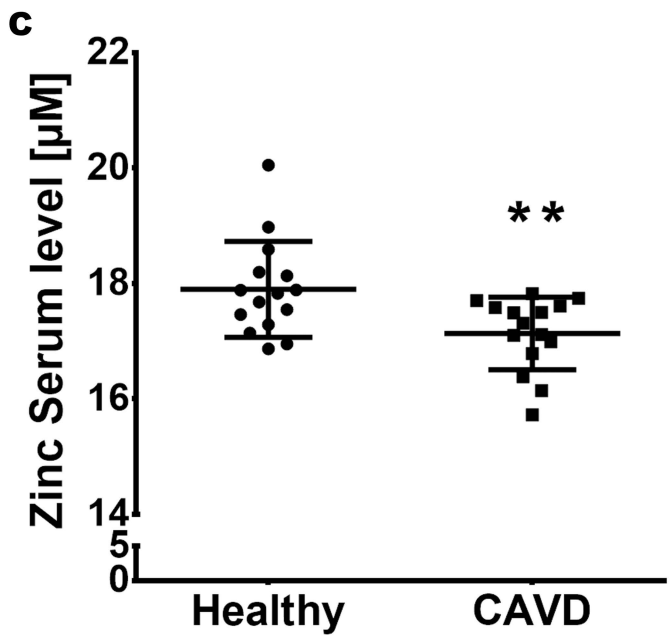

A
mRNA expression (Fold change)
Ctr
ZnSO4 [20μM]
ZIP1
ZIP2
ZIP3
ZIP4
ZIP5
ZIP6
ZIP7
ZIP8
ZIP9
ZIP10
ZIP11
ZIP12
ZIP13
ZIP14
B
Cal
Cal+ZnSO4
Ctr
siNC
siZIP13
siNC
siZIP13
C
Calcium deposition (μg/mg protein)
Ctr
siNC
siZIP13
siNC
siZIP13
Cal
Cal + ZnSO4
D
Cal
Cal+ZnSO4
Ctr
siNC
siZIP14
siNC
siZIP14
E
Calcium deposition (μg/mg protein)
NS
Ctr
siNC
siZIP14
siNC
siZIP14
Cal
Cal+ZnSO4
F
Relative MSX2 mRNA expression
siNC
siZIP13
siZIP14
siNC
siZIP13
siZIP14
Cal
Cal+ZnSO4
G
Relative ALPL mRNA expression
siNC
siZIP13
siZIP14
siNC
siZIP13
siZIP14
Cal
Cal+ZnSO4
I
Zinc fluoresence intensity (arbitray unit)
siNC
siZIP13
siZIP14
siNC
siZIP13
siZIP14
Cal
Cal+ZnSO4
H
Cal
Cal+ZnSO4
siNC
siZIP13
siZIP14
siNC
siZIP13
siZIP14
FluoZin-3 (Day7)

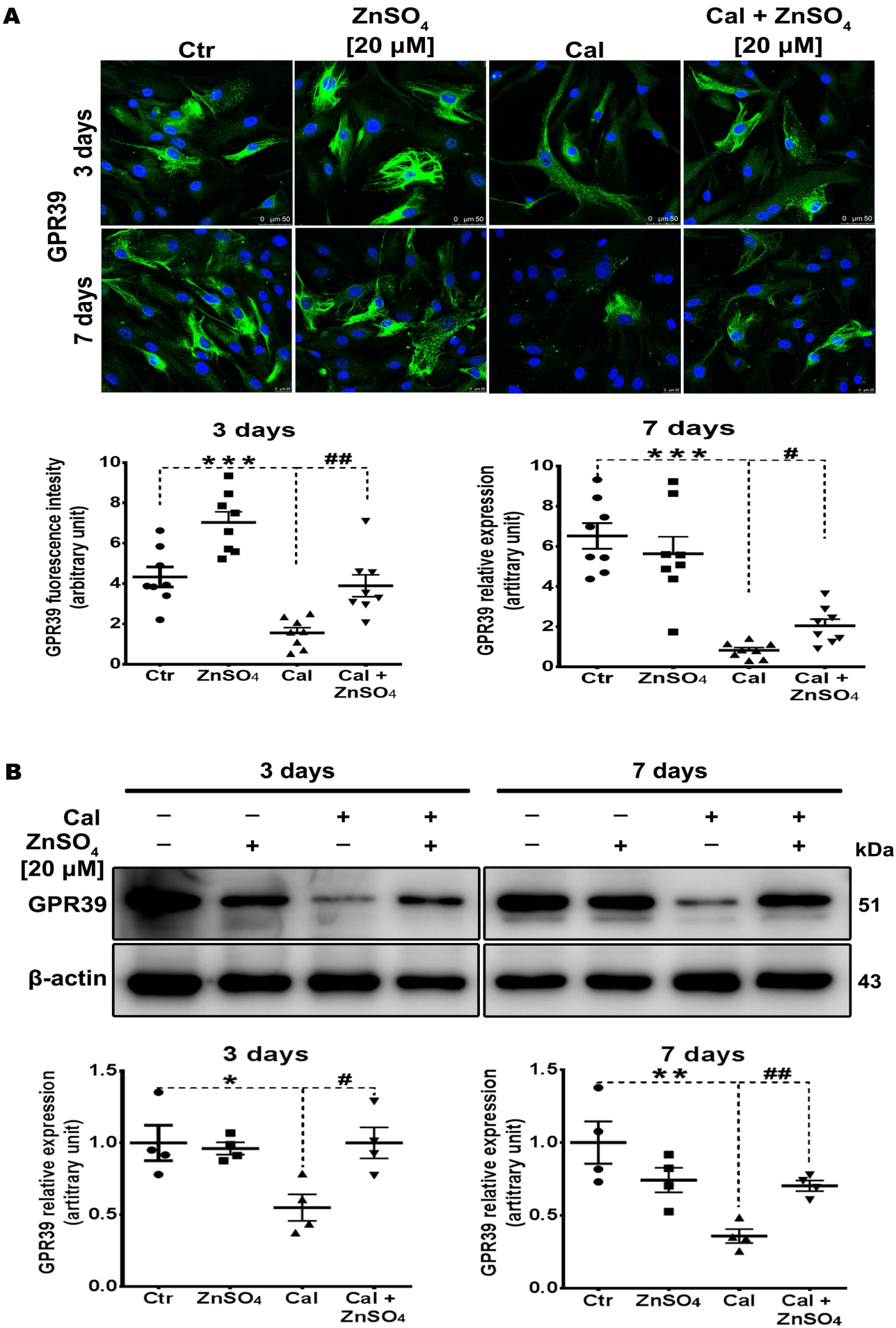
Figure 6


A
Non-Calcified
Calcified
Alizarin red S staining
GPR39
200 μm
20 μm
200 μm
20 μm


B
Non-Calcified
Calcified
1
2
3
4
5
6
7
8
kDa
GPR39
51
β-actin
43


GPR39 relative expression (arbitrary unit)
1.5
1.0
0.5
0.0
*
Non-Calcified
Calcified


c
Zinc Serum level [μM]
22
20
18
16
14
5
0
**
Healthy
CAVD


A
Ctr
ZnSO4 [20 μM]
Cal
Cal + ZnSO4 [20 μM]
GPR39
3 days
7 days
0 μm 50
3 days
GPR39 fuorescence intesity (arbitrary unit)
***
##
Ctr
ZnSO4
Cal
Cal + ZnSO4
7 days
GPR39 relative expression (artitrary unit)
***
#
Ctr
ZnSO4
Cal
Cal + ZnSO4
B
3 days
7 days
Cal
ZnSO4 [20 μM]
kDa
GPR39
51
β-actin
43
3 days
GPR39 relative expression (artitrary unit)
*
#
Ctr
ZnSO4
Cal
Cal + ZnSO4
7 days
GPR39 relative expression (artitrary unit)
**
##
Ctr
ZnSO4
Cal
Cal + ZnSO4